A MULTI-LIFE STAGE COMPARISON OF EMERGING CONTAMINANT TOXICITY ON THE EARLY DEVELOPMENT OF THREE CANADIAN FISHES

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DAYNA RAE SCHULTZ

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Canada

Chair of the Toxicology Graduate Program

Toxicology Centre

University of Saskatchewan

44 Campus Drive

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ABSTRACT

In recent years, emerging contaminants (ECs) have gained notoriety due to their increasing presence in aquatic environments as well as the lack of data available regarding their toxicity to wildlife and humans. ECs of concern such as silver nanoparticles (Ag NPs) and fluoxetine (Prozac™; FLX) primarily enter the aquatic environment as mixtures through municipal wastewater effluents (MWWEs). MWWE, which is typically a combination of industrial, commercial, and household wastes, may be released into receiving waters with little to no treatment. Such practices are not uncommon, especially in rural Canadian municipalities. While a few Canadian studies utilized rainbow trout as the test organisms for toxicological effects of select ECs, the majority of data on the effects of these compounds in fish has been garnered using laboratory species non-native to northern climates, which may not be particularly relevant considering the potential role of life history, trophic level, physiology, and climate on the species-specific toxicity of chemicals. Consequently, inaccurate extrapolation from common laboratory species to species native to northern ecosystems and with commercial, recreational, and aboriginal importance (CRA species) is concerning, as it represents a significant uncertainty factor in ecological risk assessment.

This study was completed as part of a larger collaborative effort that aimed to generate novel knowledge and approaches for the extrapolation and characterization of EC-exposed CRA fishes from classic *in vivo* studies to contemporary *in vitro* studies. This is an important and necessary step that aims to co-validate the results of each study in the overarching project, as well as begin to build a method that will be able to reliably predict *in vivo* results from *in vitro* assays. Overall, the intent is to reduce the number of live animals required for toxicity tests of ECs, as well as lessen the superfluous efforts that many *in vivo* exposures entail. For the present studies,

exposures were performed as continuous, multi-life stage bioassays for 124, 196, and 73 days with rainbow trout (*Oncorhynchus mykiss),* lake trout (*Salvelinus namaycush),* and northern pike (*Esox lucius),* respectively. Subsamples were taken at hatch, swim-up, and at the time of approximate sexual differentiation to determine potential effects on development, growth, and survival. Data on life stage-specific mortalities were analyzed together and separately in order to examine overall survival dynamics as well as to pinpoint differences in sensitivities between early life stages (ELS).

As a whole, the data obtained will contribute to the development of more appropriate environmental risk assessment strategies for North American fishes to ECs of concern. Primarily, the results show that there were clear species- and life stage-specific differences for both chemicals analyzed. For Ag NP-exposed fish, endpoints during the embryonic stage were the most frequently affected, at lower concentrations, compared to larval and fry stages. Moreover, while mortality within life stages was not generally affected, the cumulative survival across life stages was significantly lower for all treatment groups in rainbow trout and the lowest (0.10 nM), highest (30.0 nM), and middle (1.00, 3.00 nM) concentrations for northern pike. Unfortunately, cumulative survival in lake trout could not be analyzed during the last life-stage due to complications associated with the novelty of lake trout culturing in the facility. Furthermore, certain endpoints, especially degree-days to hatch and swim-up, were consistently affected across species and life stages and seemed to be the most reliable when indicating exposure to ECs. Alternately, for FLX-treated fish, the larval stage appeared to be the most sensitive, as there was clear endpoint- and species-specific outcomes. Fork length appeared to be the most sensitive endpoint for rainbow trout exposed to FLX, with the lowest observable effect concentrations (LOAECs) for embryonic and larval stages being 2.0 and 0.5 μg/L, respectively. Lake trout

depicted a significant hormetic response in fork length during the larval stage with a significant decrease at 125 μg/L. Lake trout also showed an increase in degree-days to transition to swim-up at 0.5 μg/L. Northern pike only exhibited significant effects in degree-days to hatch and swim-up, with embryonic and larval responses occurring at 125 and 500 μg/L, respectively. Moreover, both rainbow trout and lake trout demonstrated significant mortalities at 125 and 500 μg/L. There was a significant decrease in hepatosomatic index (HSI) in lake trout fry exposed to 2.00 μg/L of FLX, and histological analysis revealed increased hepatocyte volume index (HVI) in northern pike fry at 125 μg/L. For both Ag NPs and FLX, rainbow trout, which are a common and standard laboratory species used in toxicity tests for Canadian water guidelines, was either the most or equally sensitive at environmentally relevant concentrations (*i.e.* the lowest concentrations implemented). The results of this thesis suggest that rainbow trout seem to be an adequate representative species for both northern pike and lake trout with regard to assessing the toxicity of FLX and Ag NPs.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Micrograms
µg/g	Micrograms per gram
µg/L	Micrograms per litre
5-HT	5-hydroxytryptamine
Ag	Silver
Ag NPs	Silver nanoparticles
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATRF	Aquatic Toxicology Research Facility
CAS	Chemical Abstracts Service
CCME	Canadian Council of Ministers of the Environment
cm	Centimetre
CRA	Commercial, Recreational, and Aboriginal
dd	Degree days in Celsius
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
dpf	Days post-fertilization
dph	Days post-hatch
EC	Emerging contaminant
EC_{50}	Effective concentration for 50% of a population
ELS	Early life stage
FLX	Fluoxetine
g	Gram
GSI	Gonadosomatic index

h	Hour
HSI	Hepatosomatic index
ICP-MS	Inductively coupled plasma mass spectrometry
IQR	Interquartile range
kg	Kilogram
km	Kilometre
K_{ow}	Octanol-water partition coefficient
L	Litre
L/kg	Litres per kilogram
LC_{50}	Lethal concentration for 50% of a population
LC-MS	Liquid chromatography-mass spectrometry
LKT	Lake trout
LOAEC	Lowest observable adverse effect concentration
LOD	Level of detection
MATC	Maximum acceptable toxicant concentration
mm	Millimetre
MS-222	Ethyl 3-aminobenzoate methanesulfonate
MWWE	Municipal wastewater effluent
ng	Nanograms
ng/L	Nanograms per litre
nm	Nanometres
nM	Nanomolar
NOAEC	No observable adverse effect concentration
norFLX	NorFluoxetine
NP	Northern pike
ppb	Part per billion
ppm	Part per million
RBT	rainbow trout

ROS	Reactive oxygen species
SEM	Standard error of the mean
SK	Saskatchewan
SSRI	Selective serotonin reuptake inhibitor
TCDD	2,3,7,8-etrachlorodibenzodioxin
TPH	Tryptophan hydroxylase
USA	United States of America
USD	United States dollar
US EPA	United States Environmental Protection Agency

Chapter 1 **: General introduction**

1.1 Preface

The following thesis focuses on the characterization of aqueous exposures of two emerging contaminants during the early life stages of rainbow trout (*Oncorhynchus mykiss),* lake trout (*Salvelinus namaycush),* and northern pike (*Esox lucius).* Exposures commenced immediately after fertilization and continued until the approximate time of sexual differentiation. This research was undertaken in order to identify life stage- and species- specific differences, particularly with regards to important – and often poorly characterized – species native to North American freshwater systems. Endpoints for each life stage included mortality, rate of development (as degree-days to life stage transition), fork length, and hepatosomatic index (where applicable). To evaluate effects on larval morphology, survival analysis and histopathological assessment of the gill secondary lamellae and liver were used. Histopathological analyses employed length to width ratios and hepatocyte volume indices for gill and liver assessments, respectively.

This work has been organized in the manuscript style: therefore, there is repetition amongst Chapter 2 and 3 with regards to materials and methodology. Meanwhile, Chapter 1 acts as the general introduction and is intended to set the premise for the following chapters by presenting the available data on the chemicals of concern and study species used. Chapters 2 and 3 are arranged as manuscripts for publication in the Journal of Aquatic Toxicology, a peer-reviewed scientific journal. Chapter 4 is a general discussion, and thus combines the findings of Chapters 2 and 3 and outlines the implications of the present work.

1.2 Emerging Contaminants in the Environment

Emerging contaminants (ECs) are chemicals that do not hold regulatory status within Canada, yet they have been shown or are suspected to affect people as well as the environment (Deblonde et al., 2011). Currently, concern from the public and legislators over the release of ECs – which includes categories of chemicals such as nanoparticles, flame retardants, and pharmaceuticals – is increasing. Moreover, many ECs are chemicals that have been on the market for, in some instances, several decades and yet the full range of their toxic effects have yet to be adequately characterized. This lack of information is especially true for cold-water fish species native to northern environments. Many ECs are currently discharged into the aquatic environment in large amounts via wastewater effluents and run-off that could potentially lead to adverse effects in aquatic biota, as well as non-aquatic species. With municipal wastewater effluents (MWWE) being one of the primary sources for ECs, there is significant concern that common wastewater treatment technologies cannot sufficiently deactivate and/or detoxify many of these compounds which results in their subsequent and continuous discharge into the aquatic environment. This is particularly relevant for populous areas of the country with high-volume discharges as well as extensive riverine systems which may travel northward through pristine and ecologically susceptible regions. Unfortunately, these areas of high outflow often overlap with areas of high species rarity indices and conservation priority (Chu et al., 2003). While little is known about these species' vulnerability to ECs, the impact that these chemicals may have on native fish species of commercial, aboriginal, and recreational (CRA) relevance is of significant concern.

1.3 Municipal Wastewater Effluent

MWWE can be described as the cast-off water that is generated from industry, commercial, agricultural, and domestic households as well as storm, urban, and agricultural runoff that subsequently makes its way through the sewer systems (Canadian Council of Ministers of the Environment (CCME) 2006; Tetreault et al., 2011). The waste is then released directly into receiving waters or treated using various methods individually or in combination. These methods include preliminary, primary, secondary, and tertiary or quaternary treatments and include methods such as sewage lagoons, nutrient removal, and disinfection, (CCME 2006). Unfortunately, treatment type in Canada can range from no treatment or physical screening to high quality tertiary and quaternary treatments, which translates to very little uniformity not only within the country, but also within smaller geographical regions (CCME 2006).

MWWE is one of the largest anthropogenic discharges into waterways and boasting almost 3 trillion litres of effluent being discharged each year into surface waters in Canada alone (CCME 2006; Brown et al., 2011). Ensconced in the wastewater effluent mixture is human and other organic waste, nutrients, pathogens, microorganisms, suspended solids, and household and industrial chemicals not removed by treatment processes (CCME 2006). It is not surprising then that in the early 2000s across Europe and North America, greater than 80 compounds, including a plethora of pharmaceuticals and drug metabolites, were detected in aqueous environments (Herberer 2002). However, this number has presumably increased in the past ten years due to the surge in drugs on the market as well as increasing prescriptions (Heberer 2002). Unfortunately, only some of these compounds have been shown to be efficiently reduced by municipal wastewater treatment plants (Lishman et al., 2006).

In Canada, there is currently no federally enforced legislation concerning wastewater discharge (CCME 2006). Paired with the fact that the chemicals present in wastewaters are largely unknown or uncharacterized in non-target organisms and that effects on receiving environments are unclear, these discharge practices are concerning. As a complex mixture, MWWE has the potential to illicit a wide range of effects on exposed organisms and environments and, owing to the site-specific abiotic and biotic aspects of each receiving environment and the potential change in MWWE composition after treatment (if any), the potential effects are not easily generalizable (Brown et al., 2011).

Regardless of these complexities, MWWE has been shown to cause eutrophication, long and short-term toxicity, and endocrine disruption in aquatic organisms (Brown et al., 2011). Galus et al. (2013) found that, irrespective of the specific contaminants, MWWE was consistently correlated with certain effects in zebrafish (*Danio rerio*) including decreased reproductive capacity, atretic oocytes, altered morphology of the kidney proximal tubule, developmental abnormalities (at high concentrations), increased production of stress hormones, impaired gonadal development, and intersex males. Galus et al. also suggested that these effects, which to date have largely been associated with exposures to hormone analogues in the effluents, could be caused by chemicals that have not been previously linked with endocrine disruption and instead have mechanisms of action that are not mediated by steroid receptors but instead obstruct alternate pathways that ultimately interfere with the endocrine system and lead to cascade effects. Unfortunately, high quality treatments alone may not be enough to mitigate the effects of MWWE on aquatic ecosystems and can even disrupt nutrient and energy flow within food webs and thus impacting fish communities (Brown et al., 2011). Even in certain instances, ozonation, which has been shown to reduce the negative effects of some pharmaceuticals, can lead to the

formation or aldehydes of other reactive metabolites that may interfere with normal fish development (Heberer 2002; Maletz et al., 2013; Yan et al., 2014). Therefore, even in cities where tertiary or quaternary treatments are available, there may still be potential adverse effects on receiving environments, which requires further characterization and elucidation of effects of ECs on aquatic life.

1.4 Silver Nanoparticles

Silver nanoparticles (Ag NPs) can be described as conglomerations of oligomeric clusters of ultrafine silver atoms that have dimensions between 1 – 100 nanometers in at least one dimension and contain approximately 200 – 15 000 silver atoms (Wijnhoven et al., 2009; Fabrega et al., 2011). Nanoparticles can be synthesized into various shapes, such as spheres, rods, tubes, wires, multifacets, and films, to name a few (Wijnhoven et al., 2009). Furthermore, with billions of USD being invested into their development, they are being touted as an important innovation in various areas owing to their antibacterial properties, especially in the medical field (Wijnhoven et al., 2009; Scown et al., 2010). Alas, this high efficacy as antimicrobial agents also translates into a high risk for exposed biota and non-target organisms. Moreover, owing to the unique optical, catalytic, sensing, and antibacterial properties of Ag NPs and variety of uses, they are also being manufactured in increasing amounts and are currently utilized in a wide range of other areas, including the environmental, agriculture, and consumer product sectors (Wijnhoven et al., 2009; Sharma et al., 2014). Common goods that contain Ag NPs include personal care products, health care supplies, food packaging and supplements, odour-resistant products and textiles, electronics and household appliances, as well as cleaning

agents (Wijnhoven et al., 2009; Scown et al., 2010). Currently, Ag NPs are the most extensively used nanoparticles and, of the approximately 1,015 consumer products that contain engineered nanoparticles, approximately 259 utilize Ag NPs (Fabrega et al., 2011; Yang et al., 2014). However, Ag NPs are also thought to be one of the most toxic of the currently engineered nanoparticles in production (Wu et al., 2010).

Estimates of global production of Ag NPs approximate 500 tonnes per annum and are expected to increase; with this, considerable additional amounts of Ag NPs are predicted to enter the environment (Fabrega et al., 2011). And, although mammals seem to be resistant to low dose, long-term treatments of Ag NPs, other organisms, such as fish, may be more susceptible (Wijnhoven et al., 2009). Therefore, concern is rising over production volumes, their highly effective design for their intended use, and the obscure effects they may be exerting on the environment and non-target biota they are introduced to (Wijnhoven et al., 2009). In 2010, an initiative aptly named the Minimum Info on Nanoparticle Characterization was created in order to more adequately characterize nanoparticles and accurately uncover their potential adverse effects in humans and the environment, especially aquatic organisms (Scown et al., 2010).

1.4.1 ***Sources of Ag NPs***

Ag NPs may enter the environment through direct and indirect pathways and are expected to enter air, water, and sediment primarily via degradation of products containing Ag NP in landfills, accidental and unexpected release from factories, and run-off through the use of paints, cleaning agents, and sprays (Scown et al., 2010). In the aquatic environment, major inputs to surface waters include atmospheric deposition, leaching of Ag NP from landfills, discharge from wastewater treatment plants, and transport from reservoir waters to surface waters (Scown

et al., 2010). Prior to the creation of Ag NPs, elemental Ag entered the environment mainly via natural weathering of soils and rocks, or as a by-product of mining activities (Wijnhoven et al., 2009). Presently, a large number of products are being marketed that contain Ag NPs. Various medical devices and instruments are produced that are coated or impregnated with nanoparticles (such as gauze, bandages, catheters), as well as consumer products such as socks and other garments, where the Ag NPs aid in fighting odour-causing bacteria (Wijnhoven et al., 2009). However, such garments have been found to lose the majority of Ag NPs after the first few washes, after which they can make their way into surface waters (Wijnhoven et al., 2009). Along with the plethora of other products that contain Ag NPs, there is no doubt that a large fraction of this nanosilver will eventually make its way into the environment, although the exact concentrations attributable to Ag NPs is a topic that is as elusive as it is complex. However, it has been suggested, with the use of material flow analysis, that the majority of Ag NPs released from consumer products are entering sewer systems and will end up in wastewater treatment plants (Yang et al., 2014). Current modeling estimates, which were generated for European systems and were obtained using predicted environmental concentrations (PECs), of Ag NPs in freshwater systems is 0.03 µg/L in realistic scenarios and may reach as high as 0.08 µg/L in high emission scenarios, although other studies predict environmental concentrations to be in the ng/L range (Mueller and Nowack 2008; Scown et al., 2010; Mitrano et al., 2014). Unfortunately, due to the complex nature of Ag NPs and the lack of reliable methods to measure them, environmental concentrations are still widely unidentified.

1.4.2 ***Biochemistry***

Fabrega et al. (2011) describes the process of Ag NP synthesis as relatively simple and inexpensive and includes reducing elemental silver (CAS: 7440-22-4), which is commonly done via colloidal chemical reduction of Ag salts using borohydride, citrate, ascorbate, or other reductants, to reduce Ag ions and form silver atoms (Ag^0). These silver atoms then aggregate to form clusters that make colloidal Ag NPs. Usually, a particle stabilizer, also called a capping agent, is added to the solution during synthesis and is used to control particle aggregation, growth, as well as the shape of the nanoparticles (Fabrega et al., 2011). Depending on experimental conditions, such as interaction kinetics with reducing agents and absorption of the stabilizing agent onto the nanoparticles, the size, morphology, stability, and other properties of nanoparticles are flexible. Although this process is common, there are other methods of creating nanoparticles, such as synthesis in organic solvents, high temperature synthesis, and top-down manufacturing with the latter two resulting in powdered Ag NPs (Fabrega et al., 2011). Because there are various methods and reagents that can be used, there is understandably high variability between size, shape, and structure of synthesized Ag NPs with different methods, as well as those synthesized using the same methods. These variables then make detection of Ag NPs in environmental compartments extremely difficult.

Interestingly, Ag NPs seem to behave differently than elemental silver, which is why they are a current area of concern (Scown et al., 2010). Although the mechanism in which they exert antimicrobial effects can be attributed to the slow release of monovalent Ag, which then interferes with bacterial cell membranes that leads to death, previously described effects seem to be attributed to something more than the released silver ions or the capping agent, which indicates the Ag NPs themselves are inducing these effects, and therefore have biochemical

properties distinct from elemental silver (Fabrega et al., 2011). Furthermore, very little is known about the biochemistry of Ag NPs, largely owing to the staggering differences that are found in size and surface area, surface chemistry (zeta potential, crystal structure type, or surface reactivity between particles), type of capping agent/coating used, charge and aggregation/dispersal tendencies, presence or absence of functional groups, photochemistry, particle chemistry and solubility shape, preparation methods, as well as the environmental factors that the Ag NP will be found in, such as the presence of other compounds and water quality parameters (Wijnhoven et al., 2009; Scown et al., 2010; Fabrega et al., 2011; Sharma et al., 2014). With such a large array of factors that can influence the biochemistry of Ag NPs, it is almost impossible to accurately outline specific characteristics and predict how these complex particles will partition in the environment and also potentially affect biota.

1.4.3 ***Silver Nanoparticles in Aquatic Environments***

The aquatic environment is a frequent sink and likely susceptible to the effects of NPs, and Ag NPs are no exception (Scown et al., 2010). Alas, information on current levels of nanoparticles in the aquatic environment is scarce and although advancements are being made in detection methods, environmental levels are limited to approximations. This is further obscured by the fact that Ag NPs can partition to different compartments, depending upon their characteristics (Fabrega et al., 2011). One study estimated that concentrations in freshwater systems are approximately 30 ng/L (Scown et al., 2010). In sewage sludge, where most of the Ag NPs tend to congregate, one study estimated levels in the range of 1.8 – 105 ng/L, whereas another suggested approximately 9,000 ng/L (Kwok et al., 2012; Yang et al., 2014). However,

the implications of sewage concentrations for water and riverine systems has yet to be fully deciphered due to the dilution that occurs after discharge.

In aquatic environments, Ag NPs tend to aggregate with other compounds to form colloids, although this is highly dependent on the specific characteristics of the Ag NP as well as water parameters (Scown et al., 2010). Some water parameters that affect aggregation potential include cation concentration, pH, the presence of dissolved organic carbon, and humic and fulvic acids (Scown et al., 2010). Particle aggregation could lead to reduced transport of NPs in marine and estuarine environments but could also mean that Ag NPs may act as co-transporters for other aqueous pollutants (Scown et al., 2010). On the contrary, Cumberland and Lead (2009) found that Ag NPs have the potential to have long residence times in the presence of humic substances, which could potentially increase their bioavailability. Furthermore, particle size is known to influence parameters such as accumulation and partitioning in biota (Scown et al. 2010). Water characteristics such as those previously mentioned have been found to alter Ag NP size and thus lead to changes in uptake and partitioning in biota (Cumberland and Lead 2009; Kwok et al., 2012).

Although specific behaviours of different Ag NPs in the aquatic environment have yet to be characterized, studies have discovered worrisome effects caused by NPs in organisms. Yang et al. (2014) found that the compositional species makeup of microbial communities found in treated sewage, which are important for clarification and recycling of sludge and wastewater, are altered after exposure. This is likely due to the generation of reactive oxygen species in the microorganisms and bacteria, although estuarine sediment microorganisms seem to be more resistant, which could be attributed to the creation of aggregates in this highly ionic water (Scown et al., 2010). In non-fish species, Ag NPs have been seen to be taken up into plants, alter

embryo development in eastern oysters (*Crassostrea virginica),* and accumulate in the common water flea (*Daphnia magna),* which suggests that biomagnification may be a concern (Griffitt et al., 2013; Sharma et al., 2014).

1.4.4 ***Mechanisms of Toxicity***

The small size and large surface area to volume ratio of NPs are undoubtedly cause for concern and play a major role in toxicity as the size allows them to pass biological membranes while surface area to volume ratio increases reactivity (Wijnhoven et al., 2009). Ion transporters are not a likely source of incorporation and in NPs of approximately 50 nm, uptake into cells is achieved more readily due to endocytosis (Powers et al., 2010; Shaw and Handy 2011). It has been demonstrated that size allows for Ag NPs to pass through the chorion pore channels of fish, via Brownian diffusion, and inhabit the cytoplasm where they have the ability to interfere with normal developmental processes (Lee et al., 2007; Powers et al., 2011). This incorporation likely leads to previously demonstrated defects in embryonic development. In later life stage fishes, uptake occurs via the water column, sediment, or diet, and therefore the primary sites of exposure are via gills, epithelial cells, or the gut lumen, respectively (Scown et al., 2010). Therefore, neurobehavioural effects may be due to the particles passing through the blood-brain barrier to induce changes. Such neurobehavioural effects include altered swimming activity in response to light exposure in zebrafish (*Danio rerio)* after short term exposure (Powers et al., 2011). Another study, which was carried out at high concentrations in early life stage Japanese medaka (*Oryzias latipes)* for 70 days, found that exposure to Ag NPs resulted in a decrease in the optic tectum, which is an indicator of midbrain development, in a concentration-dependent manner while another found Ag NPs in the midbrain itself (Wu et al., 2010; Kwok et al., 2012).

The small size of Ag NPs would also explain the tendency of Ag NPs to accumulate in gills, liver, midbrain, gut lumen, yolk sac, blood, heart, and carcass tissue (Scown et al., 2010; Kwok et al., 2012; Griffitt et al., 2013). Unfortunately, specific toxicokinetic and toxicodynamic information is still lacking.

Once taken up into the body, studies have suggested that Ag NPs may exert effects by directly damaging cell membranes, disrupting ATP production and DNA replication, altering gene expression, releasing toxic silver ions to induce effects, or by producing reactive oxygen species (Sharma et al., 2014). Many studies have been conducted on the antimicrobial effects of Ag NPs on microorganisms and various other aquatic organisms. Toxic effects have been attributed to the creation of ROS, likely due to the emanation of silver ions (Sharma et al., 2014). This is backed up by studies that have detected increased catalase activity in organisms exposed to Ag NPs, which is associated with ROS detoxification (Shaw and Handy 2011). General edema is also a common outcome and is thought to be caused by osmoregulatory disruption (Wu et al., 2010).

Multiple studies have found that Ag NPs exert significant effects on embryo development in fish and have the potential to affect skeletal, notochord, and cardiac development, cause edema, and in some cases, affect optic development (Lee et al., 2007; Scown et al., 2010; Wu et al., 2010; Shaw and Handy 2011; Kwok et al., 2012; Griffitt et al., 2013). Lee et al. (2007) found that the critical concentration in zebrafish is 0.19 nM, after which abnormalities begin to manifest at a significant rate and only dead or deformed larvae occur. In the study, they documented spinal and tail abnormalities at concentrations of 0.04 to 0.71 nM Ag NP. At higher concentrations (0.07- 0.71 nM), they noted instances of cardiac malformations and yolk sac edema. In the 0.44 – 0.71 nM concentrations, they documented head edema and eye deformities,

which occasionally meant a complete lack of eye, retina, or lens development. These instances led to death. Findings by Lee et al. (2007) suggest that certain developmental pathways may be co-regulated and are particularly sensitive to Ag NP exposure. Kwok et al. (2012), who studied the effects of Ag NP coating in early life stage exposures of Japanese medaka, also documented an increase in embryonic malformations and pericardial edema. Although the incidences varied between coating materials and NP size, spinal flexure was a significant effect, while pericardial edema, which led to death after 24 h in most cases, was also significant. Furthermore, Wu et al. (2010) conducted a study in early life stages of medaka for a longer exposure time and found retarded ocular development along with decreased pigmentation, as well as edema, fin fold abnormalities, and heart malformations.

While some studies have found congruent effect outcomes between NP types and species, others disagree. A few studies found that Ag NP exposure resulted in delayed hatching in zebrafish, whereas other studies in medaka reported early hatching (Scown et al., 2010; Wu et al., 2010; Powers et al., 2011). There is also disagreement as to whether bulk Ag or Ag NPs are more toxic to aquatic species and whether or not Ag NPs cause significant effects on growth parameters (Wu et al., 2010; Shaw and Handy 2011; Kwok et al., 2012; Batchelor-McAuley et al., 2014). These inconsistencies could be due to species differences but are also likely due to the low number of studies exploring Ag NPs as well as the contradictions between all of the chemical parameters used, as previously mentioned. Regardless of the aforementioned incongruities, there seems to be a consensus that these effects are resultant of Ag NPs as opposed to the release of elemental silver from the Ag NP surface.

It is therefore prudent for research to continue to attempt to characterize Ag NPs in order to understand the toxicokinetic and toxicodynamic aspects that influence how they react and

partition in the environment and in biological systems. To do so, it is vital for researchers to fully elucidate the characteristics of the nanoparticles in question as well as the water parameters being used.

1.5 Fluoxetine Hydrochloride

Fluoxetine hydrochloride (FLX), which is also commercially known as Prozac™, is a pharmaceutical that is most notably used to treat psychological disorders such as depression but may also be used to treat other neurological dysfunctions such as compulsive behaviour and personality disorders, among others (Brooks et al., 2003; Kreke and Dietrich 2008). It is one of the most common selective serotonin re-uptake inhibitor (SSRI) drugs on the market. The neurotransmitter serotonin, also known as 5-hydroxytryptamine (5-HT), is likely one of the most effective and pervasive neuromodulators in vertebrates (Brooks et al., 2003). Furthermore, FLX use has steadily been rising since its inception in the mid 1980s (Kreke and Dietrich 2008; Gao et al., 2017). Like many other pharmaceuticals, FLX must reach a steady-state in the human body in order to induce effects, and therefore, requires long-term administration (Kreke and Dietrich 2008). Once in the body, FLX is metabolized to norfluoxetine (norFLX), which is a physiologically active metabolite that induces effects and is subsequently excreted from the body via the urine (Kreke and Dietrich 2008). Since wastewater treatment plants are not adequately equipped with technologies to remove these metabolites or parent compounds, which results in effluents containing products that are not or only partially degraded, they enter the aquatic environment and have resulted in concentrations in the low ng/L range (Kreke and Dietrich 2008). Once in the aquatic environment, they are taken up by biota, which may lead to

physiological alterations, as well as influence behaviour, endocrine processes, and reproductive parameters, likely resultant from the highly conserved nature of the endocrine system in vertebrates (Kreke and Dietrich 2008; Concoran et al., 2010). As a result of the increase in prescriptions, paired with long administration times and the large proportion of excess FLX and norFLX that is excreted, levels in the aquatic environment may be on the rise, which could have important implications for aquatic species (Brooks et al., 2003; Milane et al., 2006).

1.5.1 ***Sources***

SSRIs are one of the most commonly detected pharmaceuticals in surface waters and wastewater effluents, likely due to their extensive use in human medicine (Concoran et al., 2010). SSRIs and their metabolites are primarily excreted as <10% unchanged parent compound in the urine and so the major input of FLX and norFLX into the environment is via human urine, which then makes its way to wastewater treatment plants (Brooks et al., 2003; Bedner and MacCrehan 2006; Kreke and Dietrich 2008). Because there are no specific methods in place to remove FLX and norFLX, or other pharmaceuticals for that matter, from wastewater, it is discharged into the environment and is commonly detected at levels ranging from ng/L to low µg/L in water, as well as ng/g in sediment (Concoran et al., 2010). In various Canadian surface waters near sewage treatment plants, maximum levels of FLX have been measured at 99 ng/L in effluents and 46 ng/L in receiving waters (Kreke and Dietrich 2008). Similarly, in another study, sewage treatment effluent was measured to be between 99 ng/L to 841 ng/L whereas surface waters were measured at 12 ng/L to 30 ng/L (Concoran et al., 2010). Not surprisingly, the metabolite norFLX has also been detected in wastewater influents, effluents, and surface waters at concentrations reaching 9.9 ng/L in influent (Kreke and Dietrich 2008).

1.5.2 ***Biochemistry***

FLX (N-methyl-y-[4-(trifluoromethy)-phenoxy] benzenepropanamine; $C_{17}H_{18}F_3NO$) is a psychoactive drug that is made up of a racemic mixture of two lipophilic enantiomers and is non-volatile, non-biodegradable, and relatively lipophilic (Brooks et al., 2003; Gaworecki and Klaine 2008; Kreke and Dietrich 2008). FLX is a phylogenetically ancient neurotransmitter, from the synaptic cleft and thereby increasing intracellular levels (Gaworecki and Klaine 2008; Kreke and Dietrich 2008). It does this by inhibiting 5-HT transporters and by interfering with the reuptake of the neurotransmitter back into the presynaptic neuron from the synaptic cleft, which leads to a negative-feedback loop that influences firing activity (Gaworecki and Klaine 2008; Kreke and Dietrich 2008). In its solid state, the planes of the molecular constitution are defined by two aromatic rings, which are slightly skewed in their spatial orientation and hence, prevent intramolecular ring to ring interactions (Robertson et al., 1987). Furthermore, a synclinal conformation is present about the C2-C3 bond, which results in a folded, three-dimensional alignment, which is hypothesized to be necessary for the high-affinity and selectivity for the serotonin-uptake carrier (Robertson et al., 1987). It is thought that certain areas of the FLX structure may resemble serotonin, which may or may not be biochemically and pharmacologically meaningful (Robertson et al., 1987). Due to the persistence of FLX, which is ideal in human medicine in order to ensure the drug elicits effects before being biotransformed and/or excreted, it is a troublesome environmental contaminant (Gaworecki and Klaine 2008).

FLX has been widely studied in humans, but pharmacokinetic and pharmacodynamic parameters have yet to be fully elucidated in fish species although various studies have stated that species differences are likely to occur (Gaworecki and Klaine 2008; Kreke and Dietrich

2008). In humans, FLX is orally administered and induces desired effects after administration times ranging from 1 to 22 months (Kreke and Dietrich 2008). FLX will still induce effects prior to steady-state and at steady-state, the concentration of norFLX is usually greater than the concentration of FLX (Kreke and Dietrich 2008). In humans, FLX is metabolized by cytochrome P-450 isoenzymes in the liver, which results in the formation of the metabolite norFLX (Brooks et al., 2003). It is presumed that this metabolism is similar in fish species, considering the cytochrome P-450 superfamily is highly conserved among vertebrates, which could therefore display similar substrate specificity (Kreke and Dietrich 2008). FLX is up to 90% orally bioavailable, binds to plasma proteins at levels of greater than 95%, and has a large volume of distribution in the body (up to 100 L/kg body weight), whereas the half-life of FLX and norFLX are approximately 1 to 4 and 7 to 15 days, respectively (Kreke and Dietrich 2008). Furthermore, the selectivity of FLX versus other monoamine uptake carriers appears to be more than 50-fold (Robertson et al., 1987). Moreover, FLX and norFLX may have the ability to interact and inhibit some P-450 isoenzymes in fish, which may have implications when FLX is present in the environment in complex mixtures and in the presence of other contaminants (Kreke and Dietrich 2008). Consequently, FLX may have the ability to indirectly alter other signal transduction pathways in fish that may be differentially regulated, or not present, in humans (Kreke and Dietrich 2008).

1.5.3 ***FLX in Aquatic Environments***

The partitioning characteristics of FLX in the environment are lacking and it is currently unknown whether FLX or norFLX are primarily found in the water column or the sediment. Regardless, certain assumptions may be useful in predicting these important aspects on FLX

behaviour; due to the potentially high lipophilicity, it is possible that FLX and its metabolite are found sorbed to organic and inorganic materials in the water column or in the sediment (Kreke and Dietrich 2008). Furthermore, the efflux of FLX into the environment may vary seasonally due to a higher rate of societal depression in fall and winter months, which would result in higher concentrations being released into the aquatic environment during these seasons (Kreke and Dietrich 2008). With regards to wastewater treatment, FLX contains secondary aliphatic amines and is a basic drug (pKa = 10.1), so it is generally protonated at a neutral pH (Gaworecki and Klaine 2008). Along with other SSRIs, FLX tends to be resistant to the sedimentation and biological treatments that are utilized in wastewater treatment facilities and generally relied upon for decontamination of such contaminants (Gaworecki and Klaine 2008). Due to the presence of amines, FLX may be slightly broken down by chlorination and dechlorination in wastewater treatment plants; however, because dechlorination generally occurs on the order of seconds, which is inadequate for these compounds, they are more often than not released unaffected into the environment (Bedner and MacCrehan 2006).

1.5.4 ***Mechanism of Toxicity***

As previously mentioned, the toxicokinetic and toxicodynamic aspects of FLX and norFLX have yet to be fully investigated in aquatic species. In humans, FLX acts by inhibiting the 5-HT serotonin reuptake mechanism; likewise, this mechanism has been found in other vertebrates, such as fish, as well as invertebrates, although the role and mode of action in many species is not known and FLX may behave differently in fish than in humans (Kreke and Dietrich 2008). In one study, Kreke and Dietrich (2008) investigated potential endpoints in fish after FLX exposure, as well as examined the characteristics of uptake and metabolism and found that FLX

was primarily taken up across the gills and may sequester there. FLX and norFLX have also been described as vasoconstrictors, potentially having implications for the gills (Kreke and Dietrich 2008). This could lead to vasoconstriction in gills, resulting in decreased blood flow and subsequent impairment of gas exchange, which could result in chronic stress. After absorption, FLX is then incorporated into the bloodstream. Interestingly, uptake via ingestion seemed to be negligible; therefore, once in the fish body, FLX does not exhibit the first-pass effect, like it does in mammalian systems, which results in a larger fraction of the contaminant reaching receptors and inducing effects. This also suggests that mammalian systems may not be as relevant for comparison. Regardless, fish likely metabolize FLX by utilizing the same cytochrome P-450 enzyme as humans. In turn, the creation of norFLX could mean that exposure to FLX could also, like in humans, indirectly lead to compromised detoxification by inhibiting cytochrome P-450 isoenzymes. This could then result in potential effects on steroid metabolism and endocrine homeostasis (Kreke and Dietrich 2008).

After uptake, distribution, and at least some metabolism, FLX takes effect on serotonergic neurons found in the brain of fish species such as goldfish (*Carassius auratus*) and zebrafish (Kreke and Dietrich 2008). Moreover, 5-HT has been detected as a neurotransmitter not only in the fish brain, but also in other fish organs and tissues. In early mammalian embryo development, the serotonergic system is involved in processes such as cell proliferation, migration, differentiation, and morphogenetic cell movements during gastrulation and post gastrulation (Kreke and Dietrich 2008). In teleost fish development, 5-HT has been documented as having a role in growth, notochord development, locomotor behaviours, and reproductive processes (Kreke and Dietrich 2008; Airhart et al., 2012). Tryptophan hydroxylase (TPH) and monoamine oxidase, which are important in the creation and recycling of products of the

serotonergic system, have been found in northern pike, although their similarities to the mammalian analogs is uncertain (Kreke and Dietrich 2008). These findings suggest that the fish serotonergic system may be comparable to the mammalian system and could therefore be susceptible to environmental FLX exposures, resulting in increases in endogenous serotonin availability and having further effects on growth, development, and survival.

Aside from presumed mechanisms based on mammalian models, multiple studies have reported behavioural changes in fish that resulted from FLX exposure (Brooks et al., 2003; Gaworecki and Klaine 2008; Kreke and Dietrich 2008; Concoran et al., 2010). One study used a behaviourally dominant phenotype of Blue Wrasse (*Thalassoma bifasciatum*) and exposed them to FLX intraperitoneally to a single dose (10 µg/g) or multiple doses (6 µg/g for 14 days) (Kreke and Dietrich 2008). After the exposures, researchers found that both exposures lead to decreased aggression when subjected to resident-intruder tests (Kreke and Dietrich 2008). As a result, the individuals that would naturally be high on the social hierarchy scale and are presumably more evolutionarily fit, were lowered in rank. Gaworecki and Klaine (2008) also found that FLX can result in a decreased ability to catch prey in hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) at concentrations ranging from 23.2 to 100.0 µg/L but indicate that species differences, route of exposure, and concentration duration likely play a large role. Concoran et al. (2010) reviewed results that suggested FLX exposure can lead to decreased feeding rates and growth in fathead minnows (*Pimephales promelas)* at concentrations ranging from 51.0 to 170.0 µg/L and 51.0 -53.0 µg/L, respectively. Using Japanese medaka, Brooks et al. (2003) found that at levels of 0.10 to 0.50 µg/L, FLX caused increased estradiol levels as well as an increase in developmental abnormalities. Kreke and Dietrich (2008) also uncovered evidence that FLX exposure could lead to premature spawning in molluscs at levels as low as 1 µg/L and increases

in gonadotropin in goldfish and Atlantic croaker (*Micropogonias undulatus*). They also determined 48 LC_{50} values hovering around 230 μg/L for invertebrate species, and 710 μg/L and 8 900 μg/L for fathead minnows and medaka, respectively. In conclusion, data regarding effects and the biochemical reactions of FLX in the aquatic environment and species are lacking, which is likely due to the complexity of the endocrine system of aquatic organisms as well as the lack of data available on the toxicokinetics and toxicodynamics of FLX in fishes.

1.6 Model Organisms: Species of Commercial, Recreational, and Aboriginal (CRA) Importance

Fish comprise the largest and most diverse group of the chordates and have been used in almost every facet of biological research, including embryology, neurobiology, endocrinology, and environmental biology (Powers 1989). This is largely due to their usefulness in setting a precedent for preliminary vertebrate studies, and the low cost and maintenance (compared to mammalian, avian, amphibian, and reptilian models) that their care necessitates (Powers 1989; Nelson 2006). Due to their extensive use in aquaculture, there is also ample knowledge regarding the rearing conditions, as well as the developmental biology and life history, of numerous fish species which date back to upwards of one hundred years (Powers 1989).

Over the past thirty years, the use of fish in research has been steadily increasing and in Canada, fish are the most common laboratory species used across research disciplines (DeTolla et al., 1995; Casebolt et al., 1998). Due to the high magnitude of diversity between fish species with regards to habitat, life span, size, and physiological adaptations, there are a plethora of options available when choosing an ideal organism in research. Some species, such as fathead

minnow and Japanese medaka are ideal due to their short life spans and use in multigenerational studies, where others, such as zebrafish, are esteemed for their ease of use in molecular and genetic studies. Larger fish species, such as many of the salmonids, are excellent when assessing histopathological changes, owing mostly to their size (DeTolla et al., 1995; Casebolt et al., 1998). Fish of many different species have been particularly useful as indicator species and are even occasionally more acceptable when assessing carcinogenicity of chemicals than their mammalian counterparts, such as in the case of winter flounder (*Pseudopleuronectes americanus*), English sole (*Pleuronectes vetulus*), rainbow trout, and Japanese medaka (Powers 1989). Hence, due to their effectiveness in the environmental and biomedical fields, the use of fish as model organisms in research will likely remain constant.

1.6.1 ***Oncorhynchus mykiss***

1.6.1.1 As a Laboratory Model

According to Casebolt et al. (1998), there are various factors that need to be considered in order for an organism to be a viable laboratory species, which include known and attainable rearing conditions, including water quality parameters, well defined life history stages, diet, and feeding and growth rates, among other, more general parameters. Several species within the *Salmonidae* family fit these requirements and have been utilized in scientific research. Accordingly, the background information regarding rainbow trout is ample; rainbow trout was first introduced beyond its native range in 1874 and hosts a substantial amount of information regarding culture and handling procedures (MacCrimmon 1971; EC 1998). Rainbow trout, or steelhead trout in the case of anadromous populations, is a long-lived, cold-water fish

geographically intrinsic to the Pacific coast of North America from Alaska and extending into parts of Mexico, although a large portion of the population are located in the range from British Columbia to Northern California (EC 1998; Natural resources Conservation Service (NRCS) 2000). However, since global introductions began in the late 1800s, rainbow trout can now be found on every continent of the world, except for Antarctica, due in part to these introductions as well as unintentional releases (EC 1998).

Rainbow trout belong to the order Salmoniformes and family *Salmonidae* and are predominantly found in cool, pristine headwater creeks, small and large riverine systems, as well as the cooler, upper strata of lakes, estuaries, and oceans (NRCS 2000). In these environments, rainbow trout generally occupy a complex array of niches, such as undercut banks overhanging riparian vegetation, turbulent or deep waters, boulders, logs, and roots (NRCS 2000). Furthermore, they have the potential to be able to tolerate a wide range of parameters, including temperatures ranging from 0 ^{o} – 30 ^{o} C (although the extremes may only be endured for a minimal amount of time), dissolved oxygen concentrations as low as 4 ppm, and, along with the migratory steelhead, the mainly freshwater rainbow can easily tolerate saline waters (MacCrimmon 1971; NRCS 2000).

Rainbow trout are opportunistic feeders that are known to be voracious eaters and, depending on their size, will consume aquatic and terrestrial insects, aquatic invertebrates, small fish and fish eggs, as well as the offspring of birds, mammals, and reptiles (NRCS 2000; Woynarovich et al., 2011). Potentially due to their decidedly variable diets, which can often position them high in the food chain, along with their wide distribution and inclination for pristine environments, rainbow trout are touted as an important indicator species (Adams et al., 2008). Life history traits vary substantially in rainbow trout, likely owing to their fast adaptability, which suggests that

many local strains from different river systems, as well as commercial strains that have either escaped from aquaculture facilities or have been stocked in water bodies, have certain characteristics that may not be entirely conserved throughout different populations (MacCrimmon 1971; Woynarovich et al., 2011). For instance, growth rates are variable between populations and strains of rainbow trout and are often dependent upon the size of their habitat (area, depth, etc.), availability of natural food, and water temperatures, with conditions hovering around 15 ºC garnering the highest growth rates (Adams et al., 2008; Woynarovich et al., 2011). Furthermore, for populations stocked from different aquaculture origins that have been bred for hardiness, fast growth, and disease resistance, this implicates even more variability in life history characteristics (Woynarovich et al., 2011). Regardless, adult rainbow trout average approximately 30 – 45 cm and 50 – 75 cm in length for rainbow trout and steelhead trout, respectively, weigh approximately 2 – 3 kg, and reach 4 – 6 years of age (Adams et al., 2008; Jönsson 2011; Woynarovich et al., 2011). However, the oldest rainbow trout ever captured measured in at 120 cm, 25.4 kg, and was approximately 11 years of age (Woynarovich et al., 2011).

Rainbow trout reach sexual maturity between 2 and 4 years of age (Jönsson 2011). Typically, they spawn in main river channels and the associated tributaries, as well as the inlets and outlets of streams and lakes, though they most commonly use stream riffles that are located downstream from pools as their spawning area (NRCS 2000). These spawning events can either take place in spring, fall, or winter when water temperatures are optimal, although this is dependent on population (within Canada) and their geographical location (globally) (MacCrimmon 1971; Woynarovich et al., 2011). In these environments, well aerated gravel substrate is preferential; individuals of average size will select coarse underlying rock of approximately 12 to 75 mm in

size with little sediment or silt (NRCS 2000). Primarily, female trout will use their tails to dig what is known as a 'redd', which is a shallow depression in the gravel in which they lay a portion of their eggs (NRCS 2000). After the eggs are deposited, a male will fertilize them and the female will cover the embryos with substrate using her tail, and then continue to deposit eggs in other areas until her stores are exhausted (NRCS 2000). One important factor in an ideal spawning ground is sufficient water depth and minimal sediments, which increases the ability of water to percolate throughout the redd and over the embryos and acts to circulate oxygen and remove metabolic wastes associated with embryo incubation and hatching (MacCrimmon 1971; NRCS 2000).

Currently, rainbow trout are regarded as one of the top five sport fish in North America, and some even consider them the most important sport species west of the Rocky Mountains (NRCS 2000; Finn 2007). As a result of the widespread popularity of the species, their developmental biology and life history has been studied extensively (Balon 1975; Woynarovich et al., 2011). Moreover, rainbow trout have since become one of the most valued species used in commercial aquaculture and aquatic toxicology research; owing to their size, salmonid larvae and embryos have become particularly excellent models for studies of physiology and developmental biology (EC 1998; Finn, 2007; Carvan et al., 2008). Moreover, there are abundant data available that outline feeding and growth rates, as well as a fast-growing genomic database (EC 1998; Carvan et al., 2008). Furthermore, rainbow trout is a fairly hardy species with regards to tolerating a wide range of water parameters, which is highly beneficial in research settings (EC 1998). Unfortunately, rainbow trout populations have begun experiencing reduced abundance due to habitat destruction, water diversion, and point and non-point source pollution from municipal developments and agriculture (NRCS 2000; Committee on the Status of Endangered Wildlife in

Canada (COSEWIC) 2014). In the United States of America (USA), 9 different strains of steelhead trout were added to the federal endangered species list (NRCS 2000).

1.6.1.2 Development

As the water begins to reach temperatures of approximately 15 ºC, which usually occurs in spring or fall and is highly dependent upon location, rainbow trout locate suitable spawning grounds, which generally consist of shallow stream riffles, gravely substrates, and adequate water flow (NRCS 2000; Adams et al., 2008). Whereas rainbow trout generally do not migrate very far, steelhead trout can traverse upwards of 30 km per day over a couple month span (Washington Fish and Wildlife Department (WFWD) 2008). This translates into an energetically expensive journey. Moreover, rainbow trout are generally quite territorial and upon arrival at the spawning grounds, terrains are established and defended (WFWD 2008). Over the course of the spawning season, a female can release between 1,000 and 1,400 eggs per kilogram body weight, although repeat spawners may be larger and capable of producing a greater number of eggs (EC 1998). After fertilization has taken place and the embryos have been covered, incubation begins. Incubation of the embryos can take between 28 – 49 d, depending upon water temperatures, after which the embryos will remain in the redd until the yolk sac is absorbed (Adams et al., 2008). Typically, this phase, termed the eleuthero-embryo phase, is approximately two weeks, and ends with the departure from the redd and commencement of endogenous feeding and is characteristically quite prolonged in salmonids (Balon 1975; Woynarovich et al., 2011). The eleuthero-embryonic stage therefore replaces the larval stage in salmonids and is comparably longer than that of the larval phase in other species (Balon 1975). After the embryonic and eleuthero-embryonic stage, rainbow trout, along with other salmonid species, enter into the

juvenile stage, which begins with the alevin phase that lasts until complete scalation or ossification of spines (Balon 1975). Overall, development up until this stage can range between 37 to 83 days (Woynarovich et al., 2011). The alevin phase may be succeeded by the smolt or parr phase, which is denoted by the migration downstream or, in the case of steelhead trout, to the ocean, and is followed by subsequent growth (Balon 1975).

The juvenile phase occurs when fins are fully differentiated, and most temporary organs are replaced by definitive ones (Balon 1975). This stage lasts until transformation into adulthood which is marked with the maturation of gametes and is usually characterized by rapid growth and distinct colouration (Balon 1975). According to Balon (1975), the alevin, smolt/parr, and juvenile phases are all included in the juvenile stage. The juvenile stage can last anywhere from one to four years in rainbow and steelhead trout (WDFW 2008). Adulthood is marked by the first spawning event which generally occurs around three to four years and is characterized by decreased growth (Balon 1975; EC 1998). In other salmonid species, repeat spawning is uncommon, although rainbow trout have the potential to be repeat spawners (Balon 1975). The final stage of development in salmonid species is reached when growth is extensively slowed, and the condition of gametes is inferior or lacking completely; this stage is referred to as the senescent stage (Balon 1975). Furthermore, similar to the other life stages, these stages, too, are flexible and fluid between strains and populations.

In rainbow trout, biochemical signs of sexual differentiation in females have been shown to occur between 18- and 28-days post-hatch (dph; Billard 1992). Subsequently, it is not until approximately two months post-fertilization and 5 weeks post-hatch, and shortly after absorption of the yolk sac, that the initial signs of organization of primordial germ cells takes place (Billard 1992). These germ cells, which are the initial precursors to both male and female sex-specific

cells, will develop to become lamellae, which are typical structural elements of ovaries (Billard 1992). At 12 to 16 weeks of age, the association of developing germ cells into clusters and further growth of lamellae occurs, all of which will make up the layers that house future oocytes (Billard 1992). Overall, 6 months is required for the ovarian process to come to completion (Billard 1992).

The usefulness of rainbow trout in studies of northern environments is undoubtedly more relevant than many commonly used laboratory fish models, such as medaka and zebrafish, where life history, trophic level, physiology, and climate may play a crucial role in exposure to pollutants.

1.6.2 ***Esox lucius***

1.6.2.1 As a Laboratory Model

Although species in the genus *Esocidae* are not universally regarded as model laboratory fish, certain members in the family, including northern pike (*Esox lucius)*, possess many characteristics that make it a suitable candidate for use in scientific studies. Background information on northern pike is adequate, likely due to its rising popularity in aquaculture since the 1970s and, in part, due to its circumpolar range, which makes it a species of interest in northern regions worldwide (Harvey 2009). Primarily, the developmental biology and life history of northern pike has been studied extensively, with preliminary research dating back to the mid-1800s (Kunz 2004). Importantly, there are abundant data available that outline feeding and growth rates and, similar to rainbow trout, northern pike are a hardy species that can tolerate a wide range of water parameters (Frost and Kipling 1967).

Northern pike are a large, long-lived, predatory fish found in the northern hemisphere, including North America and Eurasia, in almost all freshwater systems (Harvey 2009; Department of Fisheries and Oceans (DFO) 2011). It is, in fact, one of only two species that are found circumpolar (Nelson, 2006). Pike are a ray-finned species that belongs to the order Esociformes and family Esocidae and is predominantly found in cool waters under a variety of conditions, although they prefer slow-moving waters (Harvey 2009). Pike generally inhabit thoroughly vegetated littoral zones, though their size can hinder their ability to occupy thickly vegetated regions (Wright 1990) and are able to tolerate a wide range of parameters, including temperatures ranging from 0.1^{o} – $29\ ^{o}$ C, dissolved oxygen concentrations as low as 0.3 mg/L, and are occasionally found in coastal, saline waters, denoting its ability to endure brackish habitats (DFO 2011).

Fish in the genus *Esox* are physiologically distinct from most other fish species, and Northern pike are no exception. This is due to their unique tube-shaped bodies and flat, duckbill snout. The scales of northern pike can range in depths of green with light gold, elongated spots running horizontally along the sides and back. The underside is white, and the fins are an orange/yellow colour with dark stripes. The close-set positioning of the anal and pelvic fins and a lone, deep-set dorsal fin create a streamlined morphology that allows them to reach speeds of 15 km/h, which bodes to the northern pike's predatory nature (Mecozzi 1989). Furthermore, a ring of canines lines the lower, forward jutting jaw and short, sharp, brush-like teeth line the roof of the mouth (Mecozzi 1989). These physiological adaptations are particularly useful when it comes to appeasing their large appetites. Pike, although mainly piscivorous, are opportunistic feeders and will commonly attempt to consume any organism that can fit in its mouth, including fish (even their own species), amphibians, birds, and mammals (Harvey 2009). However, due to

their decidedly variable diets, wide distribution, and high position in the food chain, pike are known to be susceptible to various bacterial and viral diseases, tumorous lesions, and the bioaccumulation of environmental toxicants including methyl mercury and multiple organochlorines (Harvey 2009; DFO 2011).

Pike are a fast-growing species and have the potential of reaching lengths of up to one metre and reach up to 30 years of age, although the majority of populations rarely have individuals over the age of 7 (DFO 2011). While pike generally grow 10 mm for every week post hatch in the Great Lakes, the average size of pike in small Canadian commercial fisheries are approximately 0.9 to 2.3 kg, where females are typically larger than males of the same age (Harvey 2009; DFO 2011). Sexual differentiation will occur around 3 weeks post hatch in *Esox*, whereas sexual maturity occurs around 3 to 5 years in females, with males generally maturing a year earlier (Lin et al., 1997; Wright 1990; DFO 2011). Once matured, spawners congregate in the spring, subsequent to the melting of the ice, and travel to highly vegetated areas, such as flooded marshes, sloughs, and wetlands, where they breed and deposit their eggs (Harvey 2009).

Due to their wide distribution and feisty nature, pike are commonly sought as a commercial and recreational fishing prize in North America and Eurasia. In Canada, commercial production of pike has been decreasing due to loss of habitat from shore developments on the Great Lakes, where the majority of commercial operations are located (Harvey 2009). Pike are quite easy to catch throughout the year and at various times of the day, although they prefer to hunt during dusk and dawn in the summer months, and throughout the day in winter months (Harvey 2009; DFO 2011). Furthermore, due to this high desirability, pike have been introduced, legally and illegally, extensively beyond its native range in other areas of the world (DFO 2011).

1.6.2.2 Development

As the ice melts from the lakes and ambient water temperatures rise, which can occur anywhere from late March to early May depending upon latitude, pike begin their migrations into vegetated wetlands, where spawning begins (Mecozzi 1989; Harvey 2009). Fertilization involves the simultaneous release of eggs (24,000 eggs per kg) and milt in a side-by-side fashion by a mating group (one female; one to three males) (Frost and Kipling 1967). The embryos are then dispersed by the parents' fin oscillations, where they then become fixed, ideally, to the surrounding vegetation by a sticky outer coat (Mecozzi 1989; Harvey 2009). By adhering to aquatic plants, the embryos are better camouflaged than those that have been dispersed in the sediment and may then be visible to predators; even so, come hatching, nearly 99% of fertilized eggs will have been consumed (Mecozzi 1989).

Frost and Kipling (1967) have thoroughly described the early physiological development of northern pike found in Lake Windermere, England, from hatching to 6 months of age. They found that after an incubation period, ranging from 14 to 21 days, depending upon water temperatures, the embryos hatch and the larvae adhere themselves to a fixed point, such as a plant, via a specially adapted suction-like appendage located near the eyes, where they will remain stuck until their yolk sac is depleted and the mouth and anus form, which occurs at approximately 5 dph, and they begin exogenous feeding. At this time, the gills have not yet developed so oxygen exchange occurs through the body surface (Harvey 2009). At 10 dph, the fry, now called alevins, have completely used up the yolk sac and can begin to feed on exogenous sources, such as zooplankton. At the same time the yolk sac is exhausted is approximately the time when the suction appendage disappears, the fry detaches, travel to the surface to fill their swim bladders, and subsequently begin to swim freely (Harvey 2009). As

fingerlings of approximately one month of age, pike will begin to feed on insect larvae and small fish as they begin to develop their expert predatory skills. Primordial germ cells start to develop in the related species, *Esox masquinongy,* at 14 mm total length, which corresponds to approximately 3 weeks post fertilization (Lin et al., 1997). Up until sexual differentiation, at 30 mm total length, which generally occurs after two weeks post-hatch, the primordial germ cells are generally indistinguishable (Lin et al., 1997; Demska-Zakes et al., 2000).

Although northern pike are not a commonly recognized model lab species (Casebolt et al., 1998), they are undoubtedly a more relevant species with regards to northern studies. Furthermore, the relatively short time to hatching, swim-up, and sexual differentiation, as well as their hardiness, makes them an ideal organism to utilize in research and multi-life stage studies.

1.6.3 ***Salvelinus namaycush***

1.6.3.1 As a Laboratory Model

Salvelinus namaycush, otherwise known as lake trout and lake char, among various other names, belongs to the *Salmonidae* family and is the largest of the char species (Redick 1967). Lake trout are stenotherms that are native to deep, oligotrophic, boreal lakes in Canada, Alaska, and the northeastern USA but have been introduced to various locations around the USA (Redick 1967; Wall and Blanchfield 2012). While there is a growing body of work delving into the life history of lake trout, it is undoubtedly less studied than other salmonids. Lake trout exhibit slow growth, late maturity (around 6-7 years depending on habitat parameters), low reproductive potential, and a slow replacement rate (not all females spawn every season) (Redick 1967; Shuter et al., 1998; Dehring and Krueger 2008). Moreover, as an important recreational and cultural

species, they are one of few major native sport fish adapted to the deep, cold waters of oligotrophic lakes (Redick 1967; Shuter et al., 1998).

Studies have shown that lake trout display an increased sensitivity to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) compared to rainbow trout which begs the question of whether this is consistent across chemical groups (Fitzsimons 1995; Spitsbergen et al., 1991). If lake trout are inherently more sensitive than rainbow trout to many contaminants, it could raise concern for their population stability, especially as a species whose populations are still impacted from sea lamprey predation and overexploitation (Redick 1967; Hansen et al. 1995). Along with a high sensitivity to one of the most worrisome groups of chemicals, lake trout are also susceptible to overfishing and invasive species; many populations that were depleted in the mid-1900s are still struggling to recover (Dehring and Krueger 2008). Furthermore, larger lake trout are top predators in their respective ecosystems, which places them at risk for bioaccumulation. While lake trout are not commonly utilized in the lab, generating a comparison between potentially sensitive species such as this and other, more well-characterized species, is a crucial step in uncovering differences in species sensitivities and fully protecting valuable ecosystems and the life they support.

1.6.3.2 Development

Lake trout are fall-spawning fishes and, unlike many salmonids, do not require running water to reproduce (Tibbits 2008). Males and females in adequate spawning condition congregate in shallow waters along rocky shores of the large oligotrophic lakes they inhabit between September to mid-November and are thought to be stimulated to spawn once water

temperatures reach approximately 12 °C (Redick 1967; Jeffries et al., 1979; Hansen et al., 1995). Lake trout never been found to spawn before fall turnover (Redick 1967). During this time, individuals in spawning condition congregate and begin spawning in early evening and night (Hansen et al., 1995). Hansen et al. (1995) found a tendency for early spawners to be smaller in size and spawn in inshore reefs, whereas larger, later spawners spawned inshore, and fish of various sizes were found to sometimes spawn in rivers and streams. Females, which can contain 6,000 eggs per 0.5 kg of body weight, may spawn several times during one season but may not reach spawning condition again for two to three years (Redick 1967). In terms of age demographics, Hansen et al. (1995) also found reports that the age of spawning lake trout in Lake Michigan was largely comprised of individuals aged 9 to 11 years old in the 1950s and ranged from 4 – 24 years in a later study in the 1970s/1980s. McDermid et al. (2010) also noted the large variation in life history in lake trout depending on geographic location and temperature, noting that colder temperatures were associated with slower prematuration growth, older age at maturity, and increased longevity. This is corroborated by a document from the British Columbia government which states that southern populations can mature as early as 5 years whereas northern populations may take upwards of 20 years to sexually mature (British Columbia Ministry of Fisheries n.d.). Therefore, fish in more northern regions would likely need to live longer to achieve a similar reproductive output, which puts them at an even higher risk of negative consequences of contaminant exposure.

After spawning, the embryos become lodged in the crevices of the substrates where they were fertilized, where they remain to incubate for 135 – 145 days (Redick 1967). The embryos then hatch, with early fry development frequently coinciding with spring melt (Jeffries et al., 1979). The newly hatched fry will remain in the shallow, rocky shores for several years and feed

primarily on invertebrates (Hansen et al., 1995). As they grow, they eventually turn piscivorous and migrate to deeper waters (Redick 1967; Hansen et al., 1995). There have been reports of significant mortality in the early life stages of Great Lakes lake trout, although it was postulated that this could potentially be due to environmental contamination as opposed to naturally low recruitment rates (Walker et al., 1991). Nevertheless, there is a need for further research owing to the potential tendency for high early life-stage mortality, sensitivity to environmental contaminants, long lives, their importance as CRA species, as well as anecdotal evidence from the Great Lakes describing slow replacement rates.

1.7 Endpoints

1.7.1 ***Survival, Growth and Rates of Development***

In high and low dose aquatic toxicity studies, survival, growth, and rates of development are commonly used endpoints to assess the toxicity of compounds (Braunbeck and Lammer 2006; Embry et al., 2010). These endpoints are generally regarded as the cumulative outcomes of cellular, molecular, and/or biochemical changes within the organism and which have also been demonstrated as reliable endpoints in determining water quality as it effects biological and health processes (McKim 1977; Woltering 1984). Furthermore, fish, while in the early life stages (especially during the period between hatching and the onset of exogenous feeding), are thought to be the most susceptible to toxic insults (Embry et al., 2010). Therefore, growth, body condition, survival, embryo viability/hatchability, developmental abnormalities, and rates of development in early life stage fish are important indicators of exposure and effects that may have implications at the community and population level (Woltering 1984). For the purposes of

this thesis, the life stage commencing at fertilization and ending at hatch is termed ‘embryos’, ‘larvae’ will refer to the life stage starting at hatch and continuing until approximate time of transition to exogenous feeding/yolk sac absorption, and ‘fry’ will refer to those individuals past the time of yolk sac absorption/transition to exogenous feeding.

1.7.2 ***Histopathological Analysis***

General water pollution is known to induce pathological changes in fish and histological analyses of tissues have been proven to be a useful tool to assess the degree of contamination, especially in sub-lethal and chronic studies (Bernet et al., 1999). Persistent biochemical and molecular changes may lead to physiological alterations and pathologies in tissues that are exposed. This could ultimately result in damages that are observable through histopathological analysis and which may manifest in reduced fitness and survivability through severe impairment to organs and tissues. Some of the most important tissues for histopathological analysis include the liver, mainly due to the significant role it plays in detoxification and biosynthetic processes, and gills, due to their proximity to the external environment and role in osmoregulation (Bernet et al., 1999; Lange et al., 2008). Furthermore, histopathological analysis is a relatively inexpensive and a straightforward alternative to biochemical biomarker analysis, although it generally takes considerably longer than other assays.

1.8 Research Objectives

Due to the lack of understanding of the potential adverse effects of ECs in North American fishes, the primary objective of this study was to determine the effects that Ag NPs and FLX

hydrochloride exert on early developmental stages (embryo, larval, fry) of rainbow trout, lake trout, and northern pike, to elucidate differences between species and life stages, and also to investigate exposures bridging multiple life stages. Very little is known about the consequences of ECs on ELS fish native to Canadian climates, especially immediately after fertilization and upon water hardening. Adequate characterization is also relevant in cold-water fish species native to northern environments, owing to the role that latitudinal differences may play on both chemical properties (such as persistence, degradation, and bioaccumulation) as well as evolutionary strategies of fishes. Many ECs are currently being discharged into the aquatic environment at constant rates and, of these, unknown amounts through wastewater effluents and other point or diffuse sources which have the potential to lead to adverse effects in aquatic biota, in particular fish. Toxic insults on native species may have particular implications to northern and rural communities and the technologies required to adequately treat wastewater are often lacking. Furthermore, the reliance on fishing as a source of income and sustenance is important to northern and rural communities. The studies undertaken here will help to elucidate the potential effects and hazards that Ag NPs and FLX may pose to survival, growth, and development of native fish species. In turn, it will add valuable knowledge to the scientific community that will potentially influence current chemical safety and wastewater regulations to more objectively and reliably protect the aquatic environment and relevant biota.

Overall Objective:

To investigate the long-term toxicity of two ECs, Ag NPs and FLX, during the early life stages (fertilization until sexual differentiation) of rainbow trout, lake trout, and northern pike

Specific Objectives:

1) Characterize effects of Ag NPs and FLX on fertilization success, embryonic development, and hatchability of rainbow trout, lake trout, and northern pike;

2) Characterize effects of Ag NPs and FLX on normal gross-morphological development of liver and gills in larval rainbow trout, lake trout, and northern pike;

3) Identify the potential impact of Ag NPs and FLX on fry survival, transition to exogenous feeding, and sexual differentiation in rainbow trout, lake trout, and northern pike;

4) Compare sensitivities of rainbow trout, lake trout, and northern pike as well as embryonic, larval, and fry life stages.

Hypotheses:

1) H0: There will be no concentration-dependent effects of the exposure to Ag NPs and FLX on fertilization success, embryonic development, hatchability, survival, or transition to exogenous feeding of rainbow trout, lake trout, and northern pike;

2) H0: There will be no differences between the sensitivities of rainbow trout, lake trout, and northern pike between species or life stages to Ag NPs and FLX;

3) H0: There will be no concentration-dependent effects on morphology, histopathology, or mortality of developing rainbow trout, lake trout, and northern pike exposed to Ag NPs and FLX.

Chapter 2 **: A multi-life stage comparison of silver nanoparticle toxicity on the early development of three Canadian fish species**

2.1 Preface

Chapter 2 focuses on the characterization of an aqueous Ag NPs exposure in the early life stages of *O. mykiss, S. namaycush*, and *E. lucius.* This was undertaken in order to identify life stage- and species-dependent differences in their sensitivity to this compound, particularly with regards to important – and often poorly-studied – species native to freshwater North American systems.

Author Contributions:

Dayna Schultz (University of Saskatchewan) designed and maintained the exposure studies, managed organisms and organized sampling events, generated and analyzed all data, and drafted the manuscript.

Song Tang (University of Saskatchewan; National Institute of Environmental Health, Chinese Center for Disease Control and Prevention) helped with experimental design, maintenance and chemical analysis of target analytes (Ag NPs)

Christie Miller (University of Lethbridge) assisted with managing exposure systems/organisms, and supported sampling events

Danielle Gagnon (University of Saskatchewan) assisted with the analysis of histological samples

David Janz (University of Saskatchewan) provided guidance for the experimental design and manuscript, as well as comments and edits.

Markus Hecker (University of Saskatchewan) provided overall guidance, advised with regard to experimental design, and provided comments and edits, as well as research funding.

2.2 Abstract

Nanoparticles are an innovative new technology that have gained notoriety due to their many uses as well as the lack of data available regarding their toxicity to wildlife and humans. To date, most data on the effects of nanoparticles on fish have been garnered using non-North American standard laboratory species and short term, high concentration toxicity tests, which may not be particularly relevant to northern species and may be resulting in inaccurate assessments of potential toxicological risks. In this study, we obtained and fertilized gametes from three fish species native to northern Canadian ecosystems (rainbow trout (*Oncorhynchus mykiss),* lake trout (*Salvelinus namaycush)* and northern pike (*Esox lucius*)) and exposed them to six incremental waterborne concentrations of silver nanoparticles (Ag NPs) beginning with environmentally relevant levels and increasing incrementally. Exposures were conducted under continuous flow-through conditions and endpoints included developmental rate (degree-days to 50% life stage transition), mortality, fork length, embryonic malformations, cumulative survival, and histological effects in gills and livers of larval fish. Results showed that there were life stage-specific differences in responses, with endpoints during the embryonic stage consistently being the most frequently affected, and at lower concentrations, compared to larval and fry stages. Moreover, while mortality within life stages was not generally affected by Ag NP exposures, the cumulative survival across life stages was significantly reduced. Degree-days to hatch and swim-up were consistently affected across species and life stages and seemed to be the most reliable when indicating potential toxicity. We also found that rainbow trout, which are an increasingly common laboratory species used in developing Canadian water quality guidelines, seemed to be an adequate representative species for lake trout and northern pike as they were equally or more

sensitive than the other species. Overall, this work will aid in the development of more appropriate environmental risk assessment strategies for CRA fishes to ECs of concern.

Keywords: Emerging Contaminants, Silver Nanoparticles, Developmental Toxicity, Freshwater Fish

2.3 Introduction

Wastewater effluents are a major point-source contributor of various emerging contaminants (ECs) into the environment. These ECs are not, or are only minimally, regulated by the government and their removal is often incomplete by conventional treatment processes (Canadian Council of Ministers of the Environment (CCME) 2006). In the aquatic environment, silver nanoparticles (Ag NPs) are a particular EC of concern that enter surface waters through atmospheric deposition, leaching, and degradation of Ag NP-containing products from landfills, discharge from wastewater treatment plants, accidental and unexpected release from factories, run-off through the use of paints, cleaning agents, and sprays into surface waters (Scown et al., 2010). Ag NPs are highly reactive substances that can be synthesized into conglomerations with varying characteristics, all of which are likely to affect toxicity as well as environmental fate (Wijnhoven et al., 2009). Due to the unique optical, catalytic, sensing, and antibacterial properties of Ag NPs, they are being manufactured in increasing amounts and are currently utilized in a wide range of areas, including the medical, environmental health, agricultural, and consumer product sectors (Sharma et al., 2014; Wijnhoven et al., 2009). Currently, Ag NPs are the most extensively used NP and are thought to be one of the most toxic of the engineered nanoparticles (Wu et al., 2010; Fabrega et al., 2011; Yang et al., 2014).

Due to the large and increasing number of products that contain Ag NPs, environmental concentrations are likely increasing (Wijnhoven et al., 2009). The exact environmental concentrations are unknown although recent predicted environmental concentrations (PECs) generated from European freshwater systems approximate 30 ng/L in realistic scenarios that may reach as high as 80 ng/L in high emission scenarios (Mueller and Nowack 2008). However, other studies predict environmental concentrations to be in the ng/L range (Scown et al., 2010; Mitrano

et al., 2014). Regardless, it has been suggested that the majority of Ag NPs released from consumer products will end up in wastewater treatment plants (Yang et al., 2014). Current estimates of Ag NPs levels in sewage reaching wastewater treatment plants ranged from 1.8 – 105 ng/L (Scown et al., 2010; Yang et al., 2014). And, due to the complex nature of Ag NPs as well as geographic- and population-dependent differences in their use, modeling of environmental concentrations is the primary means of estimating exposure (Gottschalk et al., 2009; Scown et al., 2010; Fabrega et al., 2011; Yang et al., 2014). Currently, Ag NPs hold no regulatory status within Canada, yet multiple studies suggest that they have the potential to lead to negative outcomes on the health of humans and wildlife (Ahamed et al., 2008; Asharani et al., 2008, 2009; Deblonde et al., 2011).

The small size and large surface area to volume ratio of these chemicals are both the reason for their efficacy but also their toxicity as it allows them to pass through biological membranes (Wijnhoven et al., 2009). Specifically, it has been demonstrated that size allows for Ag NPs of approximately 50 nm to pass through the chorion pore channels of zebrafish (*Danio rerio*) embryos, via Brownian diffusion, and inhabit the cytoplasm where they have the ability to interfere with normal developmental processes (Lee et al., 2007; Powers et al., 2011). However, the lower and upper size range at diffusion occurs has not been investigated. In exposures with zebrafish in later life stages, uptake of Ag NPs has been attributed to absorption via the water column, sediment, or diet, and therefore is bioaccumulated into the body via gills, epithelial cells, or the gut lumen, respectively (Scown et al., 2010). Once ingested, little is known about the further metabolism and excretion (Wijnhoven et al., 2009; Kwok et al., 2012; Griffitt et al., 2013). The small size of Ag NPs would also explain the tendency of Ag NPs to accumulate in gills, liver, midbrain, gut lumen, yolk sac, blood, heart, and carcass tissue (Scown et al., 2010;

Kwok et al., 2012; Griffitt et al., 2013). Unfortunately, specific toxicokinetic and toxicodynamic information is mostly lacking for Ag NPs.

Many studies have been conducted on the effects of Ag NPs on microorganisms and have found that microbial communities exposed to Ag NPs may be at particular risk. Furthermore, effects of Ag NPs have been documented in various aquatic organisms including microalgae (*Pseudokirchneriella subcapitata)*, rainbow trout, spiny dogfish (*Squalus acanthias)*, zebrafish, nematodes (*Caenorhabditis elegans)*, water fleas (*Ceriodaphnia dubia)*, and ragworms (*Nereis diversicolor)* have been shown to be affected by Ag NPs at a range of concentrations (Sharma et al., 2014). Studies have suggested that, once taken up into the body, Ag NPs may exert effects by directly damaging cell membranes, disrupting ATP production and DNA replication, altering gene expression, releasing toxic silver ions, or by producing reactive oxygen species (ROS) (Sharma et al., 2014). Moreover, as global production of Ag NPs rises, considerable amounts will enter sewage systems as runoff or industrial and domestic waste; subsequently, they are likely to end up in wastewater treatment plants, be released in wastewater effluents, and expose important receptors in the aquatic environment, such as fish (Scown et al., 2010; Fabrega et al., 2011). Studies have detected increased catalase activity in zebrafish and common carp (*Cyprinus carpio*) exposed to Ag NPs, which is associated with ROS detoxification (Shaw and Handy 2011). Edema is also a common outcome of Ag NP exposure in zebrafish and is thought to be caused by osmoregulatory disruption (Wu et al., 2010). Multiple studies have found that Ag NPs exert significant effects on fish embryo development and have the potential to affect skeletal, notochord, and cardiac development, cause edema, and in some cases, affect optic development (Lee et al., 2007; Scown et al., 2010; Wu et al., 2010; Shaw and Handy 2011; Kwok et al., 2012; Griffitt et al., 2013). Ag NPs have also been found to lead to neurobehavioural effects in

zebrafish, including altered swim activity in response to light exposure after short term exposure (Powers et al., 2011). Another study, which was carried out at high concentrations (100-1000 g/L) in early life stage Japanese medaka for 70 days, found that exposure to Ag NPs resulted in a decrease in the optic tectum, which is an indicator of midbrain development, in a concentration dependent manner while another found Ag NPs in the midbrain itself (Wu et al., 2010; Kwok et al., 2012).

While some studies have found congruent outcomes, others have shown conflicting responses of the exposure of fish species to Ag NPs. Some studies have found that Ag NP exposure resulted in delayed hatching in zebrafish, whereas others in medaka reported early hatching (Scown et al., 2010; Wu et al., 2010; Powers et al., 2011). There is also disagreement as to whether bulk silver or Ag NPs are more toxic to aquatic species and whether or not the NP causes significant effects on growth parameters (Wu et al., 2010; Shaw and Handy 2011; Kwok et al., 2012; Batchelor-McAuley et al., 2014). These inconsistencies could be due to species differences but are also likely due to the low number of studies exploring the toxicity of these chemicals as well as the contradictions between Ag NP parameters, including size, shape, and coating type. It is therefore pertinent to study the effects of nanoparticles on a wider range of fish species, especially those that are not as commonly isolated for laboratory research, in order to further identify the potential adverse impacts that silver nanoparticles may be eliciting in the environment.

Canada is one of the top freshwater reservoirs in the world and is also home to approximately 1,200 species of freshwater fish that exhibit diverse life history traits and ecosystem functions (Kenchington et al., 2013). Species of commercial, recreational, and aboriginal concern (CRA species), or species that support CRA fisheries, are integral

components of the ecosystem and, therefore, there is a desire to ensure their health and fitness (Kenchington et al., 2013). Many CRA species commonly demonstrate long lives, slow growth, and slow regeneration times (relative to other commonly utilized laboratory species). Due to these biological traits, early exposures to ECs such as Ag NPs in such species have the potential to negatively affect populations if the responses are severe enough early in life. Therefore, adequate characterization of the toxicological risks of these chemicals is highly relevant, especially owing to the role that latitudinal differences may play in context with both chemical properties (such as persistence, degradation, bioaccumulation) and evolutionary strategies. Toxic insults on native species may have particular implications in northern and rural communities, where much of the waste from more populated areas flows to, the technologies required to adequately treat wastewater are often lacking, and the reliance on fishing as a source of income and sustenance is important to maintain traditional ways of life.

Early life stages (ELS) of many aquatic species are often the most sensitive to contaminant exposure and even low concentration, long-term exposures can have immediate or latent effects on individuals and populations (Embry et al., 2010; Weis 2014). ELS of fishes include the periods from fertilization until sexual differentiation and are crucial for development, as they include stages of organogenesis, organ migration, general growth, and differentiation of the gonads.

In the current study, three CRA species (rainbow trout, northern pike, and lake trout) were used to assess the potential adverse outcomes of Ag NPs on the earliest developmental stages (embryo, larval, fry), and also to elucidate differences between species and life stages. For the purpose of this study, the life stage commencing at fertilization and ending at hatch are termed ‘embryos’, ‘larvae’ will refer to the life stage starting at hatch and continuing until approximate

time of transition to exogenous feeding/yolk sac absorption, and 'fry' will refer to those individuals past the time of yolk sac absorption/transition to exogenous feeding. Very little is known about the adverse effects of Ag NPs on ELS fish native to Canadian climates, especially immediately after fertilization, when an initial dose at water hardening may lead to adverse effects. This study will hence help to elucidate the toxic effects and potential hazards that Ag NPs may pose and, in turn, will add valuable knowledge to the scientific community that will potentially influence current chemical safety and wastewater regulations to more objectively and reliably protect the aquatic environment and relevant biota.

2.4 Materials and Methods

2.4.1 ***Chemicals***

Ag NPs were synthesized by the Department of Chemistry, University of Saskatchewan, Saskatoon, SK, Canada (courtesy of Dr. Robert Scott). In brief, stock solutions of Ag NPs (capped with polyvinylpyrrolidone (PVP), glucose and gluconate, and nitrate) were dialyzed for 24 h (< 0.1% free ions; approximately 30 – 100 nm), and prior to use in exposures diluted with water from the Aquatic Toxicology Research Facility (ATRF), University of Saskatchewan. Concentrations of dissolved Ag ions in the stock sample were quantified before the exposure began by an ICP-MS using $AgNO_3$ as a standard and where concentrations of total Ag were determined after nitric acid reduction (without centrifugation or filtering). Ag ions only made up a small portion of the solution with 99.9% in particulate form. The concentration of dissolved Ag ions released is defined as ionic Ag released from Ag NPs.

Samples of test solution were taken throughout the exposure and analyzed after the end of the bioassay. Solutions were analyzed whole (Ag_{total}) as well as after ultracentrifugation at 106,000 x g and the supernatant measured alone ($Ag_{dissolved\ ions}$; Equation 1). The stock solution and 30.0 nM concentrations were measured by atomic absorption spectroscopy (AAS; limit of detection = 0.05 μg/L) to measure the percent dissociated during the exposures. The total nanoparticulate silver was then estimated by applying the average dissociation rate (26.8% ± 2.97) to the estimated total silver concentrations.

Equation:

$$[Ag]_{particle} = [Ag]_{total} - [Ag]_{dissolved\ ions} \quad (1)$$

2.4.2 ***Fish Collection and Egg Fertilization***

Spawning lake trout and northern pike were collected in October 2015 and May 2016, respectively. Lake trout were collected from Lac la Plonge (55°08′N 107°20′W; n = 3 males and 1 female) and northern pike were collected from Lac la Ronge (55°10′N 105°00′W; n = 4 males and 2 females), both of which are clean, large, glacial lakes located in Northern Saskatchewan. Ripe males and females were collected using gill nets and placed in a holding tank for < 2 hours before gamete collection. Weights and fork lengths were measured, and eggs or milt collected by gently applying pressure to the abdomen. Male fish were released back to the environment after milt collection while female fish were either released or euthanized by blunt-force trauma for further tissue sampling. Rainbow trout gametes were obtained from Troutlodge (Sumner, WA, USA; n = 3 males and 3 females).

All materials used during egg fertilization procedures were sterilized with either 75% ethanol or 0.000075% Proviodine® (povidone-iodine; Rougier Pharma, Mirable, QC, Canada) solution. Milt from males and eggs from females were first pooled before combining. For rainbow trout and lake trout, eggs and milt were combined and mixed for approximately 2 min before activation with water (ATRF water and site water, respectively), while northern pike were gently stirred with a slurry of bentonite clay to bind and minimize mucous. For lake trout and northern pike, fertilized eggs were immediately transferred to either plastic bags or glass mason jars containing 1 L of exposure solution, which were then oxygenated and placed in coolers for transportation to the ATRF. For all species, transfer to exposure solutions occurred within an hour of fertilization Embryos were allowed to water harden in exposure solutions for at least 12 hours before being transported back to the University of Saskatchewan to ensure the initial exposure during water hardening and reduce susceptibility to mechanical damage immediately after fertilization. For rainbow trout, embryos were transferred directly to 10-L exposure tanks. Embryos were transferred randomly to exposure solutions, with each replicate containing approximately 120 embryos (rainbow trout) or 80 embryos (lake trout, northern pike).

2.4.3 ***Experimental Setup***

All exposure experiments were conducted at the Aquatic Toxicology Research Facility (ATRF) at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). The methods used herein were approved by the University of Saskatchewan's Animal Research Ethics Board (AUP #: 20140079) and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Each of the 6 test concentrations of aqueous Ag NPs (0.1, 0.3, 1.0, 3.0, 10, 30 nM) were diluted with ATRF water. Each treatment was made up of three replicate tanks with each

containing two egg chambers (cleaned and presoaked PVC pipe with a mesh bottom) to contain and separate the different life stages and allow for easy inspection. Tanks were held in a water bath that was equipped with a temperature regulator and thermometer to ensure temperatures within allowable ranges for the culturing of fish amongst treatments throughout the duration of the experimental exposure (Environment Canada (EC) 1998).

2.4.4 ***Incubations***

For rainbow trout, embryos were transferred directly to flow-through exposure tanks whereas, upon return to the facility, lake trout and northern pike were transferred into aerated 1-L glass jars and then to flow-through tanks upon reaching the eyed stage. ATRF water quality was measured daily for dissolved oxygen, pH, and conductivity, and bi-weekly for ammonia, alkalinity, and nitrates using a YSI Quatro Professional Plus multiparameter field cable (Yellow Springs, USA) or testing kits (LaMotte, Chestertown, USA), respectively. Further water quality can be found in the supplementary materials (Appendix A). Temperatures simulated environmental conditions so that ranges were between 12 and 14º C for rainbow trout, 10 and 12 º C for lake trout, and started at 10 and gradually increased to a maximum of 15 º C for northern pike. Embryos were examined daily for any occurrences of infections, morbidities and mortalities, and unfertilized and undeveloped/dead embryos, and any dead organisms were removed from the tanks. After 48 h, fertilization success was determined for all replicates (total dead embryos at 48 h/ total number of eggs · 100). Fertilization success was greater than 90% for lake trout, 75% for rainbow trout, and 98% for northern pike. Numbers of fish transitioning to the next life stage (hatch, swim-up) per day were recorded and individuals were transferred either from the embryo cup to the larval cup (after hatch) or from the larval cup into the tank

(after swim-up) in order to accurately count and determine life stage-specific endpoints. When 50% of organisms within a tank transitioned, the median time to transition was determined. After all viable organisms had transitioned to the next life stage, a sub-portion of the group was euthanized using an overdose of buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate) and immersed in 10% buffered formalin for 24 – 48 h before being transferred into 70% ethanol for storage until histological examination (in the larval stage only) of gill and liver was undertaken. During the exposure, culling was carried out as necessary in order to reduce crowding.

Apical endpoints for the embryonic stage included cumulative degree-days to 50% hatch, mortality, and fork length across all species, as well as malformations upon hatch (presence/absence of skeletal curvatures, edema, hemorrhage, craniofacial malformations) assessment in rainbow trout and lake trout only. Apical endpoints for larvae included cumulative degree-days to 50% swim-up, mortality, and fork length. After swim-up, the lowest observable adverse effect concentration (LOAEC) and no observable adverse effect concentration (NOAEC) were determined, and only LOAEC and NOAEC concentrations were carried out for the remainder of the exposure. During the fry stage, fish were fed at ad libitum with commercial start-up feed (rainbow trout and lake trout) or brine shrimp (northern pike) at least twice per day (EC 1998). After the estimated time to sexual differentiation occurred, the remaining LOAEC and NOAEC concentrations were terminated and apical endpoints, including morality, and fork length were measured. Hepatosomatic index (HSI; Equation 1) was calculated where possible (lake and rainbow trout fry) and omitted for northern pike as the body size was too small to complete dissections. Due to culturing difficulties with the lake trout, survival data past swim-up and certain apical endpoints in fry were omitted.

$$HSI = \frac{Weight_{liver}}{Weight_{total}} \quad (2)$$

Assessments of developmental deformities and growth and development in fry were conducted as outlined in the EC (1998) report on toxicity tests in early life stage salmonids, whereby embryo/alevin/fry mortality of up to 40% by the time of 50% swim-up is acceptable before the test becomes non-viable.

2.4.5 ***Histopathology***

Whole-mount fish were processed and embedded in accordance with the protocol in place at the Western College of Veterinary Medicine (Saskatoon, SK), sagittally and serial-sectioned to 5 – 7μm thickness and stained following the hematoxylin and eosin protocol in place at the Toxicology Centre (Saskatoon, SK). Evaluation of histopathologies were completed using an Olympus model BX41 microscope (Olympus America, Melville, NY, USA) with Infinity Capture and Infinity Analyze Software (Ver 6.5.4: Lumenera Corporation, Ottawa, ON, Canada). All serial sections were then thoroughly assessed for a consistently placed (same approximate position in the fish) section that lay parallel to the sectioning plane with minimal twisting or minimal artefacts, and, for gills, visibility of the entire primary lamellae. Quantitative analysis of gill morphology in rainbow trout ($n_{control}$=11, $n_{treatment}$=9) and lake trout ($n_{control}$=6, $n_{treatment}$=11) was done by measuring the height and length ratio of all (where possible) secondary lamellae across multiple primary lamellae (where possible). In the case of the liver, slides for rainbow trout ($n_{control}$=11, $n_{treatment}$=9), lake trout ($n_{control}$=6, $n_{treatment}$=12), and northern pike ($n_{control}$=6, $n_{treatment}$=6) were initially screened for evidence of alterations, after which, if histopathologies were observed, three random views of the liver (40X magnification) were selected and

hepatocyte volume index (HVI) was determined by counting the number of cells within a consistent, predetermined grid area (100 μm^2) (Palace et al., 2002). HVI analysis was used as an indicator of the cellular size of liver parenchyma in a 100 μm^2 area so that a relatively lower HVI indicated an increase in cellular size.

2.4.6 ***Statistical Analysis***

Data were tested for normality (Shapiro-Wilkes test) and equality of variance (Brown-Forsythe Test for Homogeneity of Variance) using GraphPad Prism 7 (San Diego, CA, USA). To determine significant differences between the controls and three or more treatment groups, a one-way analysis of variance (ANOVA) with post-hoc analysis (Dunnett's t-test) were performed for parametric data. Non-parametric data were analyzed using a Kruskal-Wallis H test with post-hoc analysis (Dunn's t-test). For fewer than three treatment comparisons, an unpaired t-test was used in cases of parametric data or a Mann-Whitney test (Exact p-value) on nonparametric data. Data that passed parametric assumptions are presented as mean ± one standard error of the mean (SEM), and those that did not are presented as the median ± interquartile range (25^{th} percentile, 75th percentile). All statistical tests were 2-tailed with an alpha value of 0.05. Due to sampling complications, for non-LOAEC and NOAEC concentrations, larval subsampling took place into the fry stage, and therefore, these data were used for fry life stage data, although it does not extend as far as the LOAEC and NOAEC data.

Survival data were generated using count values of mortalities obtained throughout the duration of the exposures and analyzed in Microsoft Excel®. Data from each life stage was combined with the other life stages and averaged across replicates in order to analyze cumulative

survival of each treatment. Survival analysis took into consideration all fish used in the study, including those that were culled, sampled, or that died for unexpected reasons throughout the duration of the study. Random, large numbers of deaths (*i.e.* greater than 10% of individuals seeded per life stage per replicate; not attributable to water quality) were uncommon throughout the exposures and used to identify potentially confounding factors that caused mortality instead of the exposure chemical. These data were censored in most cases except when they occurred during the life stage transitioning window, as this window is known to be a time of naturally high mortalities (Vardy et al., 2014) or when they occurred with other indicators of morbidity (listlessness, moribundity).

To determine the percent surviving on a given day ($Survival_t$), the percent surviving on the previous day ($Survival_{t-1}$) was multiplied by the proportion of the total that remained alive on the day of interest. This was obtained by subtracting the sum of dead on the day of interest ($\sum Dead_t$) from the sum of surviving on the previous day ($\sum Alive_{t-1}$) and dividing by the sum of surviving on the previous day (Equation 3).

$$Survival_t = (Survival_{t-1})\ x\ \left(\frac{\sum Alive_{t-1} - \sum Dead_t}{\sum Alive_{t-1}}\right) \quad (3)$$

2.5 Results

2.5.1 ***Water Concentrations of Ag NPs***

ATRF facility water remained within allowable ranges (dissolved oxygen > 80%; pH 7.6 – 8.0; conductivity 220 µS/cm; ammonia < 0.02 µg/L) throughout the duration of exposures. Concentrations for the stock solution and 30.0 nM concentration were measures as total silver

and dissolved ionic silver. Ionic silver was determined after ultracentrifugation (r_{av} = 106,000 x g) by measuring the silver concentration in the supernatant. These values were then used to determine the total nanoparticulate silver as well as the associated percent dissociated. The average percent dissociated for the measured solutions were used to extrapolate concentrations in the remaining treatments until analysis of all water samples is complete. Therefore, all samples other than the stock solution and 30.0 nM are estimates of expected concentrations. Preliminary results can be found in Table 2.1.

Table 2.1: Concentration of Ag NPs in exposure solutions associated with nominal concentrations (nM) and stock solution (†) where the lowest nominal concentration mimicking environmental concentrations. Total silver (ng/L) and silver concentration in supernatant after ultracentrifugation (r_{av} = 106,000 x g) were analyzed (*) for the stock and 30.0 nM concentrations using AAS and used to calculate expected total nanoparticulate silver and dissociation percentages. Dissociation percentages were then applied to nominal concentrations.

Nominal Concentration (nM)	Total Silver (ng/L)	Total Nanoparticulate Silver (ng/L)	Concentration of Dissolved Silver Ions (ng/L)	Percent Dissociated (%)
0.00	<LOD	< LOD	< LOD	n.a.
0.10	6.45	4.5	1.73	26.8
0.30	19.3	13.5	5.18	26.8
1.00	64.5	45.1	17.3	26.8
3.00	193	135	51.8	26.8
10.0	645	451	173	26.8
30.0*	1,930	1,375.0	559	28.9
30,000*†	1,690,000	1,270,000	418,000	24.7

2.5.2 ***Cumulative Survival Analysis***

Species varied in their cumulative survival dynamics when exposed to continuous, long-term exposures of environmentally relevant and subacute concentrations of Ag NPs (Figure 2.1). As previously mentioned, the life stage transition window is normally characterized by high mortality, which corresponds to days 31 – 43 and 42 – 63 days post fertilization (dpf) in rainbow trout (Figure 2.1A) while northern pike were days 1 – 22 and 14 – 24 dpf for hatch and swim-up, respectively (Figure 2.1B). However, due to culturing difficulties that occurred around the time of swim-up in lake trout, survival data had to be omitted and certain, later, life stage specific endpoints could not be analyzed accordingly.

Rainbow trout and northern pike survival both declined within the transition windows, although the degree of the decline was dependent on concentration ($p < 0.05$). Rainbow trout exhibited a clear trend with significantly greater mortality in fish exposed to Ag NPs concentrations greater than 0.10 nM relative to controls, showing an almost immediate divergence of survival in exposure groups compared to controls. Furthermore, while the controls only showed minor decreases in average survival during the transition windows, there were effects on survival where treatments higher than 0.10 nM had significantly lower survival, with divergence becoming apparent during the first transition window at hatch. Survival in all exposure groups dropped below 50% (66.4 ± 12.6% in control while treatments ranged from 37.6 ± 3.47% to 4.28 ± 2.68%) by the end of the study. Northern pike exhibited a constant decrease in survival across all treatment groups, likely owing to the overlapping transition windows. Responses were indistinguishable until approximately 7 days into the exposure when exposed fish began to show decreasing trends in survival relative to controls. This resulted in significantly different survival in the 30.0 nM (54.2 ± 2.52%) and 0.10 nM (60.5 ± 3.62%)

exposure groups, as well as the 1.00 and 3.00 nM groups (54.2 ± 3.07% and 53.9 ± 2.16%, respectively) compared to controls (73.3 ± 2.29%).

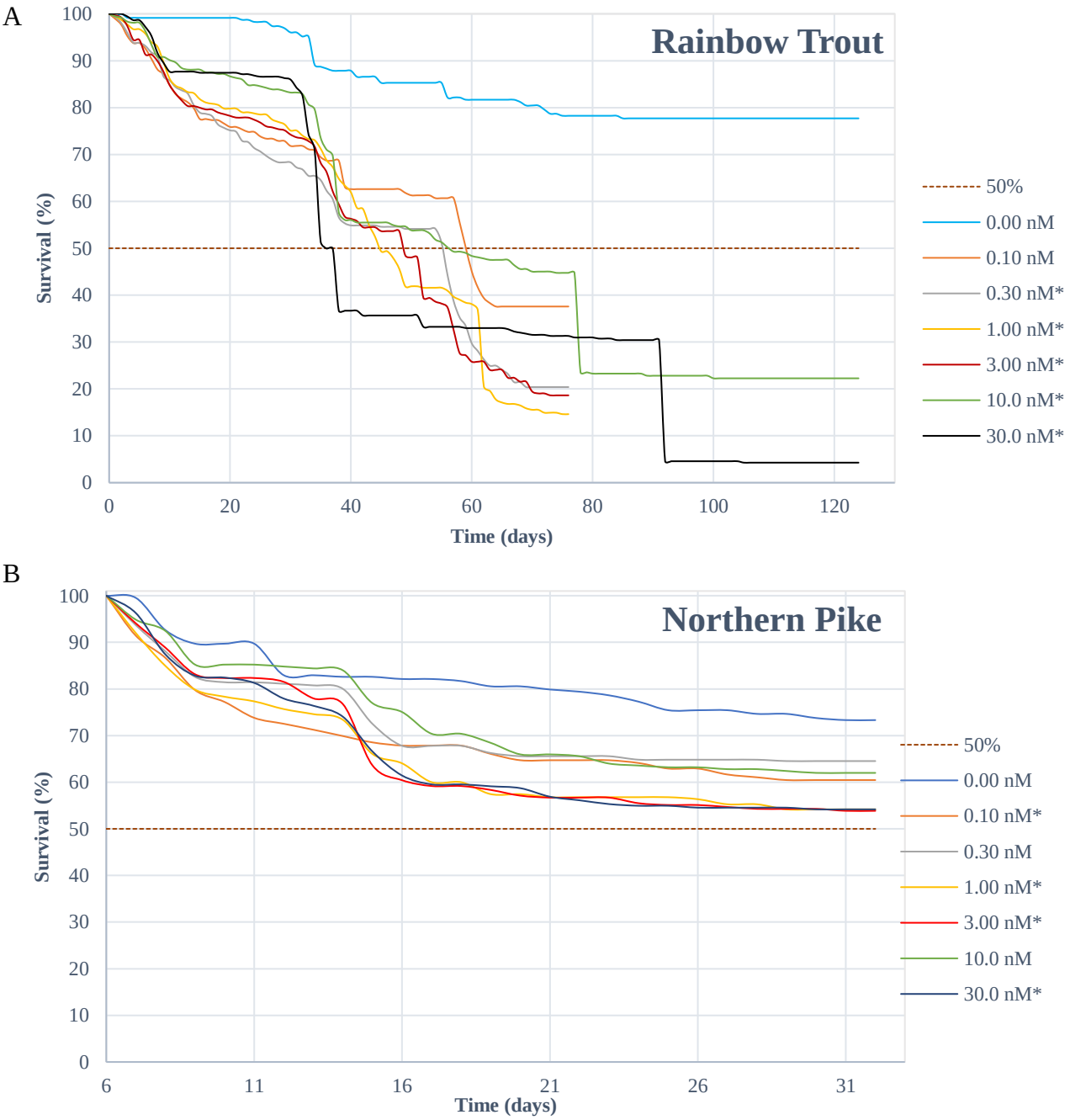


Figure 2.1: Survival analysis of rainbow trout (A) and northern pike (B) toxicity results from each treatment to give an overall treatment-specific survival curve for the Ag NP toxicity test from fertilization until approximate sexual differentiation (rainbow trout) and eyed until swim-up (northern pike). (*) indicate statistically significant differences from controls at the end of the study period ($p < 0.05$).

2.5.3 ***Apical Endpoints in Embryos***

Developmental time to hatch was significantly impacted in all three species following Ag NP exposure (Figure 2.2A). Lake trout embryos exposed to increasing concentrations of Ag NP exhibited a significantly longer developmental time at 3.00 nM (671 ± 9.75 degree-days), 10.0 nM (645 ± 6.13 degree-days), and 30.0 nM (654 ± 24.7 degree-days) than controls (573 ± 3.57 degree-days; $p < 0.005$). Rainbow trout also showed an increase in developmental time of 462 degree-days (434, 462) at 30.0 nM compared to the control median of 399 degree-days (390, 414; $p < 0.05$). Conversely, northern pike displayed a generally decreased median developmental time of 143 (129, 143) and 150 (136, 150) degree-days at 0.10 and 30.0 nM, respectively, compared to the control time of 212 (201, 224) degree-days ($p < 0.05$). Embryo mortality was not affected in lake trout or rainbow trout. Northern pike, however, demonstrated an increase in percent mortality at 3.00 nM with a median percent mortality of 37.4% (34.2, 38.6) compared to the control (22.5% (14.7, 24.5); $p < 0.05$; Figure 2.2B). However, although mortality was not significantly different at the 30.0 nM, values were higher than control in rainbow trout and northern pike. There was a concentration-dependent increase in the prevalence of malformations, such as edema, skeletal curvatures, and hemorrhage (Figure 2.3) in rainbow trout, which was significant at 30.0 nM (19.0 ± 7.12%) compared to controls (3.50 ± 1.50%; $p < 0.005$; Figure 2.2C). Moreover, there was a significant decrease in fork length of embryos at hatch in lake trout, rainbow trout, and northern pike ($p < 0.05$, 0.0001, and 0.05, respectively) at various concentrations, although the concentration-dependent effect was not consistent among treatments (Figure 2.2D). Lake trout displayed negative responses at 10.0 nM, while rainbow trout lengths were significantly affected at 0.10 and 0.30 nM. Northern pike were significantly shorter at 1.00 nM. Exposure to Ag NPs during water hardening had no effect on fertilization success in any species, percent mortality in lake trout or rainbow trout, or percent malformed in lake trout (Appendix B). It is important to note that several of the significantly

affected endpoints did not exhibit concentration-dependent responses that were consistent among treatments and that data were quite variable in some cases.

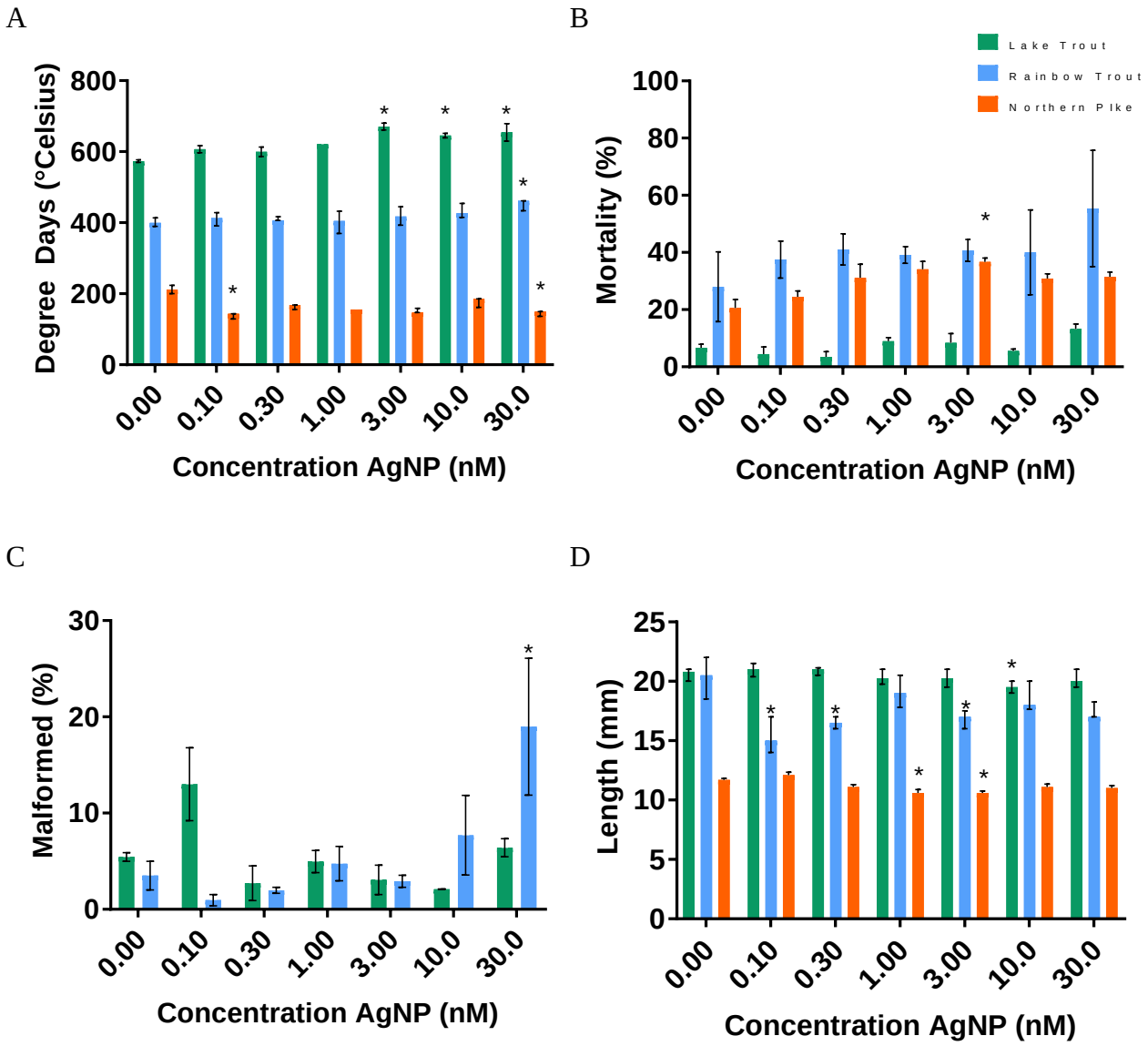


Figure 2.2: Effects of increasing concentrations of Ag NPs from fertilization to hatch of lake trout (green), rainbow trout (blue), and northern pike (orange) embryos for (A) degree-days (°C), (B) mortality (%), (C) malformations (%), and (D) length (mm). Bars represent the mean +/- SEM or median and upper and lower limit of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

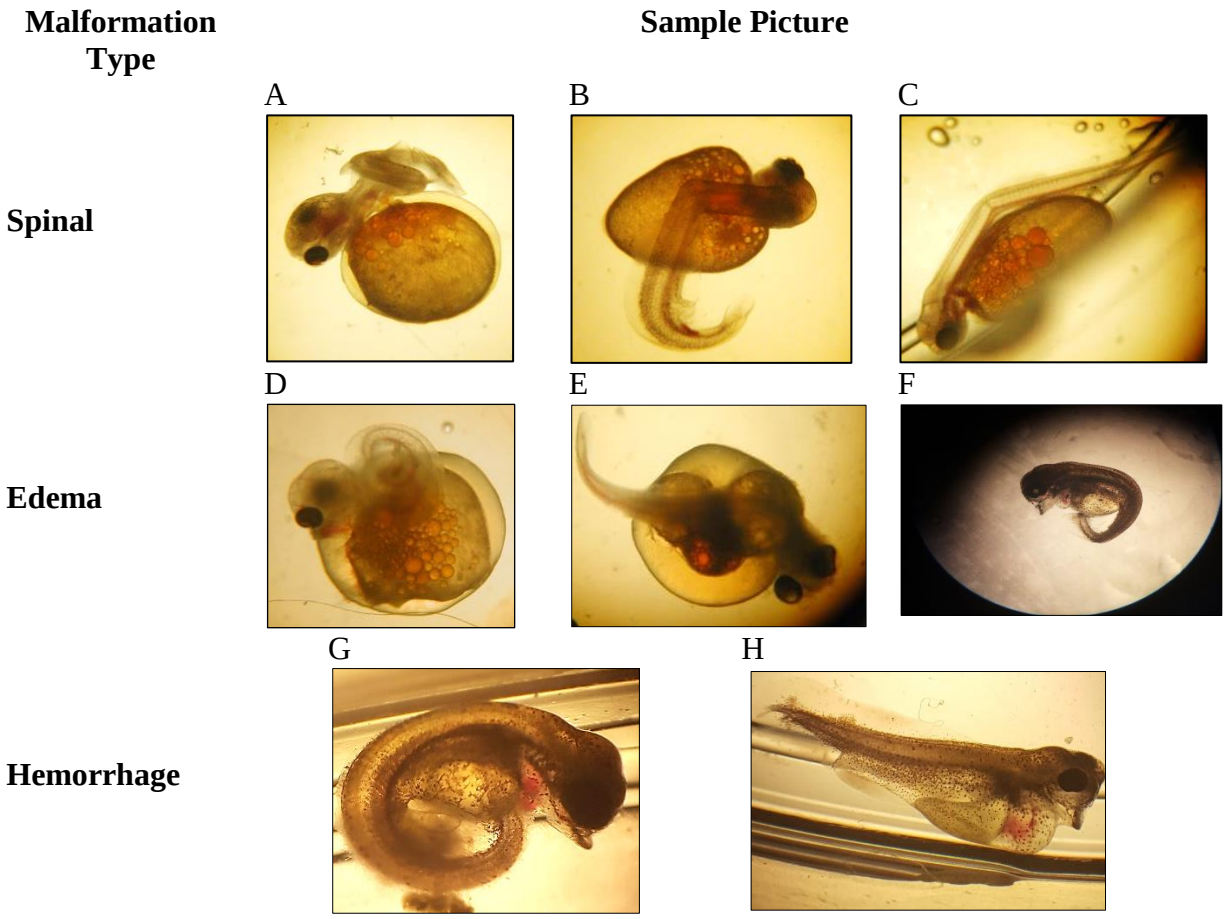


Figure 2.3: Representative pictures of common malformations present at hatch in lake trout (A, D, E), rainbow trout (B, C), and northern pike (F-H) embryos after exposure to Ag NPs.

2.5.4 ***Apical Endpoints in Larvae***

Various endpoints were negatively affected during the larval stage although, similarly to the embryonic stage, several endpoints/species did not display concentration-dependent responses (Figure 2.4). Developmental time to swim-up was once again significantly impacted in all three species and in a concentration-dependent fashion for the trout species ($p < 0.05$; Figure 2.4A). Lake trout larvae exhibited a significantly longer developmental time at 3.00 nM (488 ± 17.7 degree-days) versus controls (381 ± 5.15 degree-days). Rainbow trout also showed an increase in developmental time at 30.0 nM (252 ± 24.0 degree-days) compared to the control group (202 ± 4.73 degree-days). Similarly, northern pike displayed a generally increased median developmental time of 203 (191, 213) degree-days at 0.10 nM compared to the control time of 120 (118, 124) degree-days although data was more variable across treatments ($p < 0.005$). Larval mortality (Figure 2.4B) was again not affected in lake trout or rainbow trout but there was a significant increase in percent mortality at 30.0 nM (21.4 ± 2.02%) compared to controls (7.65 ± 1.16%) in northern pike. However, there was variability within replicates and the number of mortalities was nearing the upper allowable limits (<40%) in controls for both species. Similar to the embryonic stage, there was a significant decrease in fork length of larvae at swim-up in rainbow trout and lake trout ($p < 0.05$); lengths for larval pike were omitted due to subsampling complications (Figure 2.4C) and did not show a clear concentration-response relationship. Lake trout responded negatively at 3.00 nM with lengths of 28.2 ± 0.331 mm compared to 29.6 ± 0.491 mm in controls, while rainbow trout showed decreased lengths of 31.1 ± 0.340 and 27.7 ± 0.526 mm at 10.0 and 30.0 nM, respectively, compared to control lengths of 29.7 ± 0.339 mm ($p < 0.05$). Continuous exposure to Ag NPs showed no effect on percent mortality in lake trout or rainbow trout (Supplementary Materials).

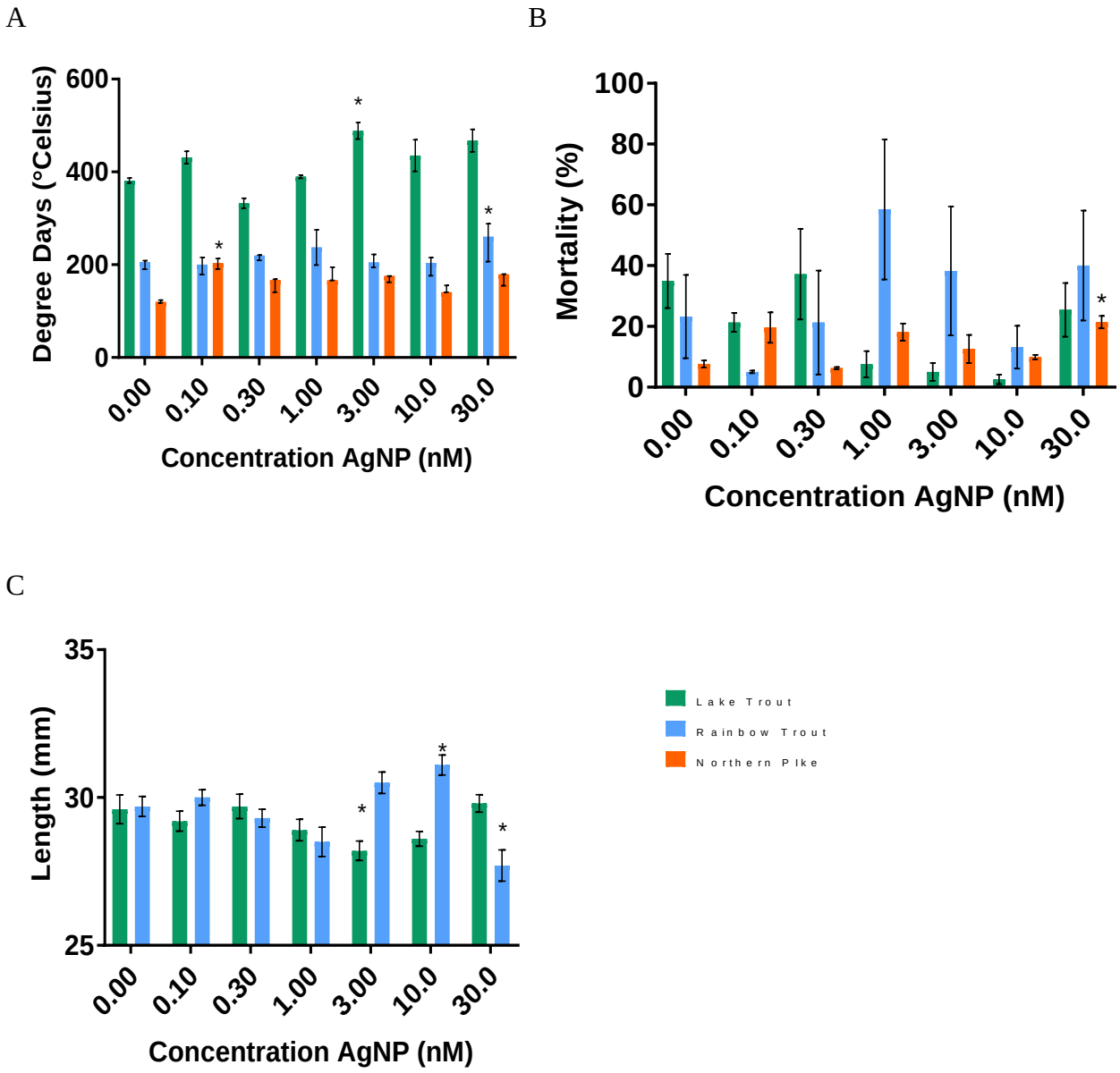


Figure 2.4: Effects of increasing concentrations of Ag NPs from hatch to swim-up of lake trout (green), rainbow trout (blue), and northern pike (orange) embryos for (A) degree-days (°C), (B) mortality (%), and (C) length (mm). Bars represent the mean +/- SEM or median and upper and lower limit of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

2.5.5 ***Apical Endpoints in Fry***

Percent mortality was not significantly different than that in control treatments in any species (Supplementary Materials). Moreover, fork length at the end of the exposure was only significantly decreased in lake trout, with lengths of 54.9 ± 0.435 mm and 56.3 ± 0.498 mm in the 1.00 and 3.00 nM treatment groups, respectively, compared to a control value of 62.0 ± 1.47 mm ($p < 0.0001$; Table 2.2). Hepatosomatic index (HSI) was measured in both rainbow trout and lake trout, although only rainbow trout exhibited a significantly increased HSI at 30.0 nM (1.59 ± 0.0851) compared to control (1.15 ± 0.0592), which indicates a relatively larger liver size ($p < 0.005$; Figure 2.5).

Table 2.2: Fork length of lake trout, rainbow trout, and northern pike fry after 208, 126, and 73 days (respectively) exposed to various concentrations of Ag NPs. Data are represented as the mean +/- SEM or median (25^{th}, 75^{th} percentile) of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

Species	Ag NP Concentration (nM)	Fork Length (mm)
Lake Trout	0.00	61.8 (58.2, 67.1)
	1.00	54.5 (52.5, 56.7) *
	3.00	56.5 (54.0, 59.0) *
Rainbow Trout	0.00	52.2 ± 0.496
	10.0	52.4 ± 0.662
	30.0	53.7 ± 0.785
Northern Pike	0.00	20.1 ± 0.554
	10.0	21.5 ± 0.483

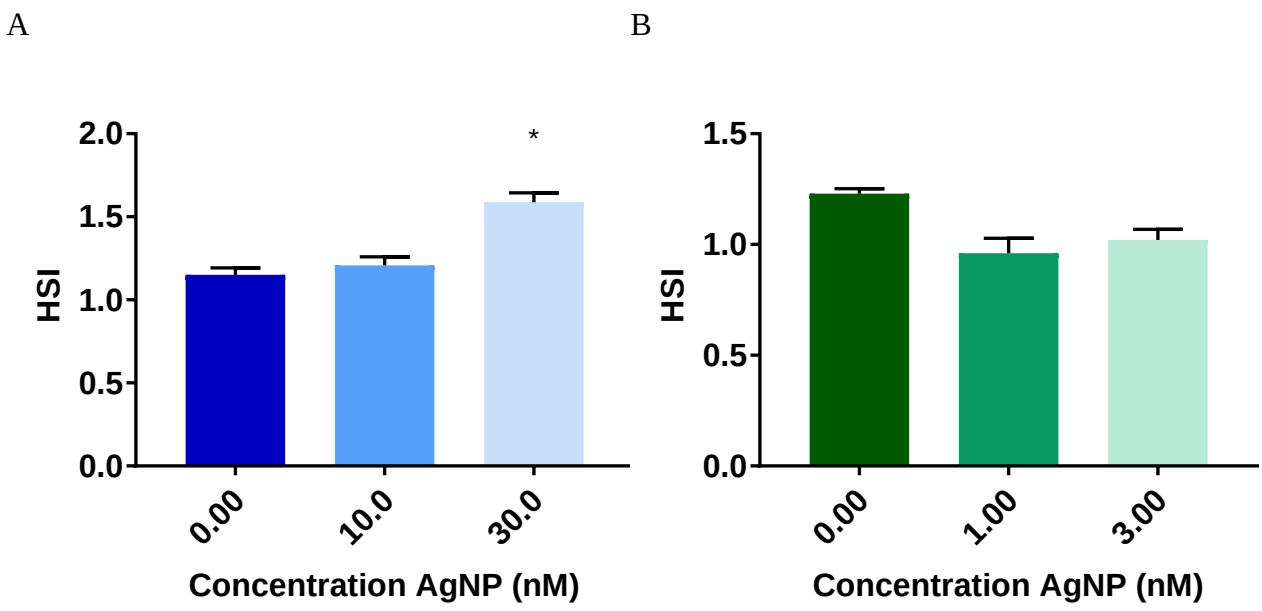
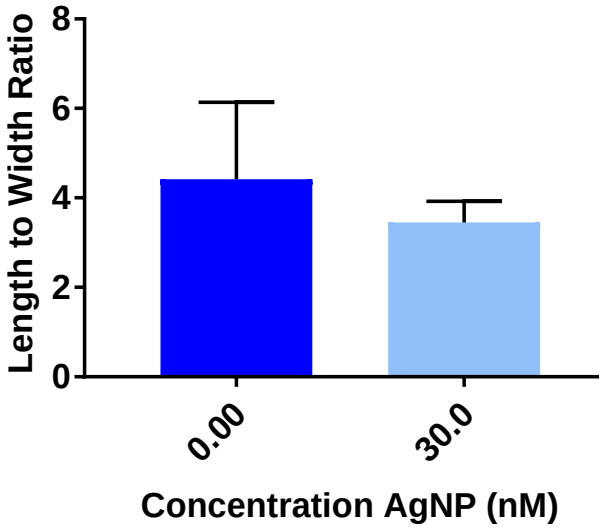


Figure 2.5: Hepatosomatic index of rainbow trout fry (A) and lake trout fry (B) after exposure to the LOAEC and NOAEC concentrations of Ag NPs. Bars represent the mean +/- SEM of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

2.5.6 ***Histopathology***

Length to width analysis of the secondary lamellae yielded no statistically significant differences among treatments in lake and rainbow trout ($p = 0.063$ and 0.061, respectively) (Figure 2.6). However, certain indicators of toxicity were noted in the gills of rainbow trout (Figure 2.7). The gill morphology of control rainbow trout was unaffected and depicted a range of normal structures of primary (PL) and secondary (SL) lamellae. Further examination in the gills of rainbow trout exposed to 30.0 nM Ag NP found instances of thickening of primary lamellae (white arrows), congestion (C), hypertrophy (HT) and shortening (LS) of the secondary lamellae, epithelial hyperplasia (HP), disruption of the cartilaginous core (*), and increased interlamellar space (LS).

A



B

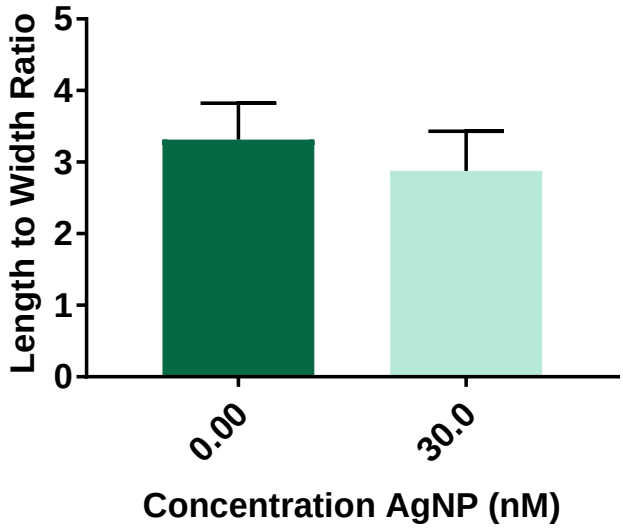


Figure 2.6: Length to width ratios of secondary lamellae in gills of rainbow trout (a) and lake trout (b) larvae after exposure to the LOAEC and NOAEC concentrations of Ag NPs. Bars represent the mean +/- SEM for replicates. Northern pike were omitted due to small size.

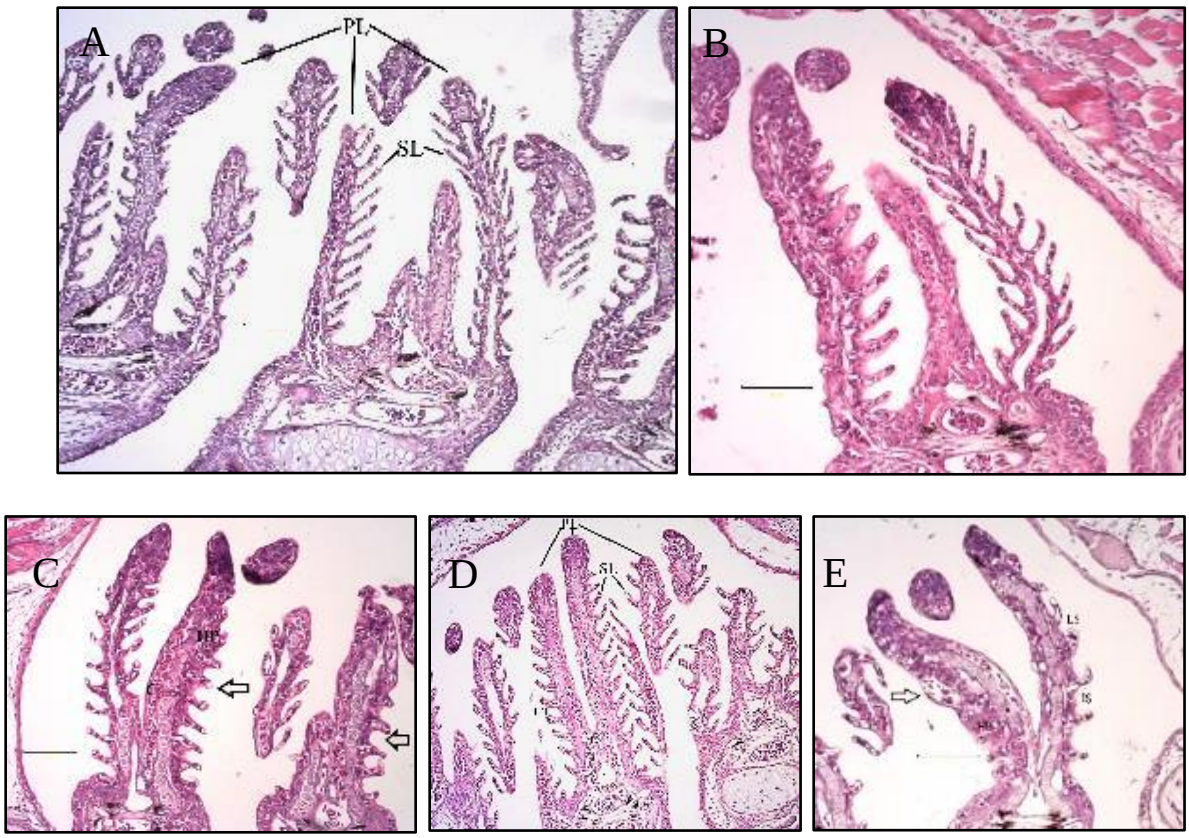


Figure 2.7: Sagittal gill sections at 100 X magnification of control (A, B) and exposed (Ag NP 30.0 nM; C-E) larval rainbow trout. Control gills demonstrate normal gill architecture, with primary lamellae (PL) of varying sizes and long, thin secondary lamellae (SL). Exposed gills (C – E) demonstrate thickening of the primary lamellae (white arrow), congestion (C), hypertrophy of secondary lamellae (HT), secondary lamellar shortening (LS), epithelial hyperplasia (HP), disruption of the cartilaginous core (*), and increased interlamellar space (IS). Bar = 100 μm.

Lake trout did not exhibit a significant difference in HVI values (control of 32.1 ± 0.694 and 30.0 nM of 34.3 ± 0.841) whereas rainbow trout displayed a significant decline in HVI from 37.9 ± 2.64 in controls to 24.0 ± 1.31 after continuous exposure to 30.0 nM of Ag NPs ($p < 0.0005$; Figure 2.8). Conversely, northern pike portrayed no significant changes in HVI with control values of 41.9 ± 2.40 and 30.0 nM HVI of 51.0 ± 3.88. Histological examination of the liver of rainbow trout showed increased lipid vacuolization, cloudy swelling (hydropic degeneration), as well as karyorrhexis and pyknotic cells (Figure 2.9).

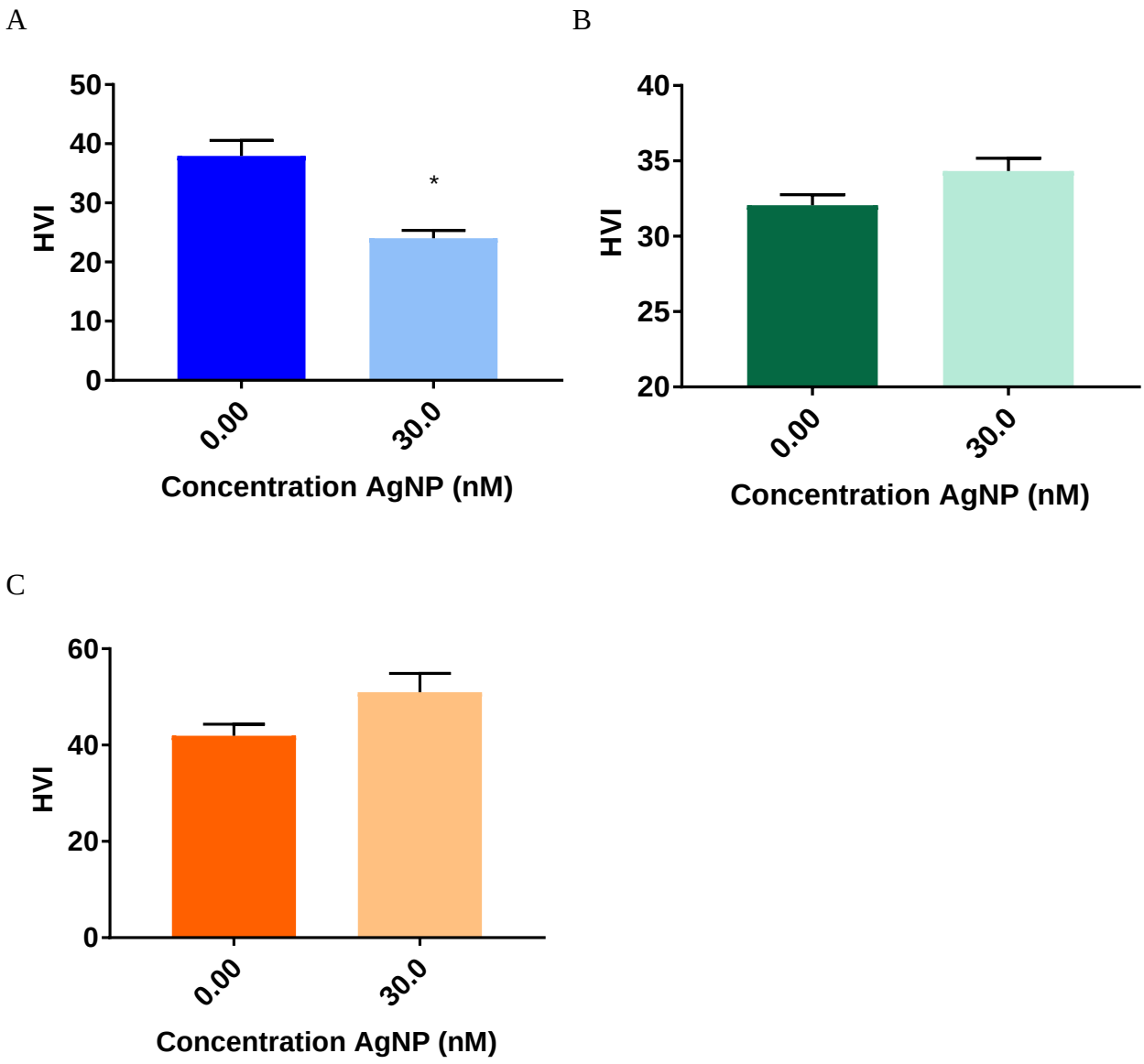


Figure 2.8: Hepatocyte volume index (HVI), as an indicator of cell size, in lake trout (A), rainbow trout (B), and northern pike (C) fry after exposure to the LOAEC concentration of Ag NPs. Bars represent the mean +/- SEM of replicates.

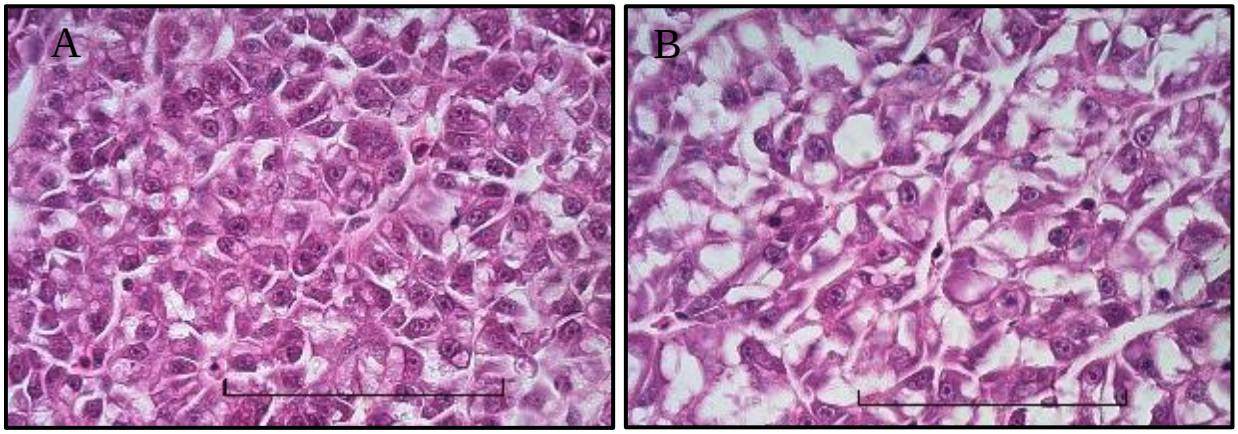


Figure 2.9: Sagittal liver sections at 400 X magnification of control (A) and exposed (Ag NP 30.0 nM; B) larval rainbow trout. Control liver demonstrates normal parenchymal architecture whereas exposed liver demonstrates increased lipid vacuolization, hypertrophy of the nucleus/nuclear swelling, and increased sinusoidal space. Bar = 100 μm.

2.6 Discussion

2.6.1 ***Survival Analysis***

Previous reports suggest that Ag NPs have the ability to pass through the chorion of developing embryos even after fertilization, which allows for the uniform uptake and distribution of the NP throughout the rapidly developing cells (Lee et al., 2007; Asharani et al., 2008; Ahamed et al., 2010; Wu et al., 2010). Subsequently, there is a high likelihood that the dispersal throughout the embryo is uniform as well (Asharani et al., 2008). Nanoparticles that are approximately 50 nm in diameter may be taken up more readily via endocytosis (Powers et al., 2011). The particles in this study ranged from 30 to 100 nm in diameter, which brackets the 50 nm value, and may have introduced uptake via slightly varying routes, although data in this field are lacking. A uniform distribution within the exposure medium, with little to no conglomeration of the nanoparticles, is then likely to cause more effects at low concentrations than the outcome of highly conglomerating particles. However, many Ag NPs, including those used here, utilize a capping agent which aims to reduce aggregating tendencies (Fabrega et al., 2011). So, capped Ag NPs are more likely to traverse the chorion as opposed to their conglomerating, uncapped, counterparts, which would not be able to pass (Fabrega et al., 2011). Regardless, significant effects on cumulative survival were observed at varying concentrations, which may suggest variances in chorion permeability among species, amount of water uptake at water hardening, or ontogeny of clearance mechanisms among these species compared to other standard laboratory species (Asharani et al., 2008, 2010). For the larval life stage, considering its brevity, there is limited literature available on the most likely mechanism of uptake of Ag NPs. After hatch, the newly emerged larvae are still developing many of their organs, including gills and scales, so uptake across skin is a potential route along with uptake from NPs already deposited in the yolk

sac (Asharani et al., 2008; Handy et al., 2008). Conversely, in fry, Ag NPs are taken up across the recently developed gills or via ingestion (Scown et al., 2010). Once the Ag NPs distribute into the developing fish body, they will exert effects by directly damaging cell membranes, altering gene expression, disrupting ATP production and DNA replication, and producing reactive oxygen species (ROS), which will subsequently lead to further damage (Sharma et al., 2014).

Unfortunately, due to the long duration of the lake trout exposure, and also potentially owing to the novelty of lake trout husbandry within the facility, a large number of control fish were lost. This resulted in a situation where a single death resulted in a disproportionate decrease in survival, which skewed results around and after swim-up. However, both rainbow trout and northern pike exhibited >60% survival in controls, as is in accordance with Environment Canada embryo/alevin/fry guidelines (EC 1998). Rainbow trout displayed significant impacts on cumulative mortality in all treatment groups even though there were no individual life stages that showed increased mortality. Mortality in northern pike was significantly affected in 1.00 and 3.00 nM treatments during the embryonic stage and at the 30.0 nM treatment during the larval stage. However, due to the nature of these exposures, it was difficult to compare values generated here to those generated elsewhere using different species. Shahbazzadeh et al. (2009) found that the 96 h LC_{50} values for juvenile rainbow trout (average weight of 1.05 g, which was similar to the rainbow trout fry here) to Ag NPs was 2,300 ng/L. Therefore, a considerably longer exposure may elicit a lower LC_{50}, which was in line with the results herein where significant decreases in mortality were observed at concentrations greater than 0.1 nM, (2.2 ng/L), although comparisons between LC_{50}s and LOAECs are difficult. However, attempting to characterize survival dynamics requires precise parameters for temperature, water quality

parameters, and other environmental factors and, so, exposure to additional stressors such as chemical contaminants at varying concentrations will further complicate the attempts to accurately and thoroughly describe survival dynamics. This is especially true for Ag NPs; the chemical characteristics are so inconsistent across studies that even modeling information of environmental concentrations is sparse. It is interesting to note that, within the first couple of weeks of the survival analysis, the survival rates already began to diverge from the controls, but significance was not detectable until much later. Furthermore, individual life stage mortalities were usually only significant for the highest treatment groups. These findings suggest that confined life stage analysis may not be robust enough to detect potentially detrimental outcomes in fish when chronic exposures that span multiple life stages are suspected. Additionally, studies that primarily focus on one life stage (especially for studies that span mere hours) may be providing false negative results that are then used to extrapolate to real-world circumstances, where exposures are more likely be long-term, low concentration exposures that encompass multiple life stages.

2.6.2 ***Apical Endpoints***

Interestingly, exposure of lake trout and rainbow trout to Ag NPs was found to cause an increase in developmental time in the 3.00 to 30.0 nM treatments and 30.0 nM treatment, respectively, which resulted in a delay in hatching. In contrast, exposures in northern pike resulted in a decreased developmental time and an earlier onset of hatching but was only significant for the 30.0 and 0.10 nM concentrations. This indicates that there were likely species-specific mechanisms and pathways involved in Ag NP developmental toxicity, whereby trout exhibited a classic concentration-dependent response whereas pike exhibited a biphasic response.

A concentration-dependent increase in developmental time was also found to occur in zebrafish exposed to increasing concentrations of Ag NPs, while another study found a decrease in time to hatch in Japanese medaka further demonstrating that the effects of Ag NPs are species-dependent (Asharani et al., 2008; Wu et al., 2010; Powers et al., 2011). The manner through which Ag NPs may interfere with hatching in fish may be through disruption of enzymatic processes that mediate hatching, which would explain the observed increase in developmental time in rainbow and lake trout, and the decreased time in pike (Korwin-Kossakowski 2012). Studies have found that a choriolytic enzyme is excreted from hatching gland cells on the head of embryos of various species (including rainbow trout and pike) as well as upper and frontal areas of the yolk sac (Korwin-Kossakowski 2012). This enzyme is made up of two proteases: a high molecular weight and a low molecular weight choriolytic enzyme, each of which aid in different aspects of the hatching process (Korwin-Kossakowski 2012). However, different species may have slight differences in the makeup of these metalloproteases, which not only could explain observed molecular weight differences but could potentially indicate variations in mechanism of action (Hagenmaier 1974). It was also found that metal ions have an inhibitory effect on the action of these proteases, which could help to explain the results seen here (Hagenmaier 1974). In embryos, there appeared to be a high number that failed to transition from embryo to larvae, *i.e.* they only partially emerged from the chorion and exhibited ruptured yolk sacs, although this endpoint was not specifically measured. This phenomenon was also noted in another study by Elonen et al. (1998) in lake herring, lake trout, brook trout, northern pike, and rainbow trout when exposed to TCDD and was hypothesized to be due to neuromuscular weakness, which could also describe the cause for the mortalities in embryos herein. Paired with the observed opposite direction of responses that Ag NPs had on hatching in trout and pike, it seems that the

proteases may have different compositions that are differentially affected by Ag NP. Interestingly, Korwin-Kossakowski (2012) found that the presence of suspended particles in water lead to more severe swelling, along with earlier hatching and reduced survival, in pike embryos. This theory could also explain the observed effects here in that, perhaps Ag NPs were becoming sorbed to the chorion and inducing effects. This could then suggest that, while trout may be adversely affected by internalization of Ag NPs, pike may be more sensitive to external Ag NPs. However, more thorough investigation is required to adequately identify the precise mechanism by which Ag NPs affect hatching in these species.

Multiple studies have found that exposure to Ag NPs can lead to a delay in swim bladder inflation, or potentially a lack of swim bladder development, which may cause a failure to transition to swim-up fry (Laban et al., 2010; Powers et al., 2010; Powers et al., 2011). It was observed here that there was a delay in time to swim-up. It is interesting to note that while only the lowest concentration of northern pike had a significantly longer developmental time, the remaining concentrations had higher values than controls. An increased sample size could then reveal that the higher concentrations did actually produce effects on development. Subsequently, differences in degree-days ranged from 22 to 60. Consequently, because northern pike are spring spawners and spawn immediately after ice-off when water temperatures reach 6 to 12 °C, this shift in degree-days could result in swim-up delays of 2 to 10 days. This could then result in a biologically significant effect considering pike are in a race to make the transition from planktivorous diets to piscivorous (and even cannibalistic) diets, which is a gape-limited factor and therefore, size-dependent (Jönsson et al., 2011). If their time for growth and development is hindered, they may be subject to an increased risk of predation (Jönsson et al., 2011). Nevertheless, higher concentrations appeared to have increased variability in developmental

time, specifically in the trout species. It is postulated that Ag NPs may interfere with the cholinergic signaling pathway by affecting neurodevelopment, although it is yet unclear whether this is due to the release of silver ions or the effects of the nanoparticle itself (Powers et al., 2010). This may have important implications due to the idea that earlier swim bladder inflation leads to a smaller mass-to-volume ratio of the larvae, and thus, decreases energy demands during a time when searching and hunting for food is important (Powers et al., 2010). The two trout species exhibited an increase in developmental time while northern pike exhibited a decreased developmental time. Lake trout are fall spawners while northern pike are spring spawners and rainbow trout can be either, depending on the population. For the trout species, this shift in developmental time could mean a decreased time span for development and growth before ice over occurs, and therefore could put them at a disadvantage for the winter, when food is lacking, and growth is slowed. On the contrary, pike are spring spawners and are thought to undergo such a rapid development, with high growth rates and succinct life stages, in order to place them at a predatory advantage compared to their commonly spring-spawning prey species (Jönsson et al., 2011). Earlier hatching and associated decreased fork length could potentially reduce their chances of growth and development in the summer months as well and have similar negative implications in reduced fitness over the winter months.

It is well known that exposure of various teleost fish species, regardless of life stage, to NPs leads to increased mortality, malformations, and impairments in growth (Lee et al., 2007; Asharani et al., 2008, 2010; Bar-Ilan et al., 2009; Ahamed et al., 2010; Chen et al., 2010; Wu et al., 2010). However, many of these studies assessed toxicity at high concentrations, with levels in the mg/L range, over the span of a few days, which creates difficulty when attempting to compare sensitivities among species here that were exposed in low μg/L concentrations over

months. Regardless, exposures of early life stages of fish to Ag NPs may lead to detrimental outcomes such as those stated above by damaging DNA directly, particularly considering a lack of cell cycle checkpoints and DNA repair pathways during such early life stages (Asharani et al., 2010). Many of the specific malformations, including defects of the eyes, circulatory system, edema, axis curvatures and truncation, were also noted here and contributed to the concentration-dependent increase in malformations in rainbow trout with a significant effect in the 30.0 nM treatment group. Literature values for malformations in other species were slightly higher, with Wu et al. (2010) noting malformations in embryonic medaka exposed to 100,000 ng/L Ag NPs, while Asharani et al. (2008, 2011) showed sensitivities in embryonic zebrafish above 5.0 $x10^7$ and 2.5 $x10^7$ ng/L Ag NPs in two separate studies, although these concentrations are much higher than those found in the environment or those used here. Notably, water chemistry data shows that dissolved silver ions in the highest treatment group was approximately at or slightly higher than the maximum acceptable toxicant concentration (MATC) level for affecting growth in rainbow trout over the long term (CCME, 2015). Regardless, endpoints were affected at ionic concentrations much below this, and therefore, the negative effects associated with these concentrations were likely due to nanoparticle toxicity.

There did not appear to be a normal concentration-dependent effect on embryo mortality in pike and no significant differences in embryo mortality in either trout species was noted, which likely indicates that the concentrations used here were below those which cause mortality. Moreover, it is difficult to make comparisons due to these aforementioned discrepancies. However, a study conducted by Wu and Zhou (2013) reported mortality in Japanese medaka embryos exposed for two weeks to Ag NPs and found a NOAEC of 0.1 mg/L, which is still substantially higher than the concentrations used here. In another study in Japanese medaka,

there was found to be a U-shaped concentration-response pattern for growth with significant changes beginning as low as 2.0 $x10^6$ ng/L, which was a similar response to that found in this study in northern pike where embryonic mortality was associated with decreased fork length as well as for rainbow trout fork length, although at concentrations of less than 1,000 ng/L (Wu et al., 2010). On the other hand, responses in larval length were inconsistent in lake and rainbow trout (no pike data available) where lake trout showed a decrease at 1.00 and 3.00 nM and rainbow trout exhibited an increase at 10.0 nM, followed by a decrease in fork length at 30.0 nM. Mortality was significantly impacted only in the 30.0 nM exposure group for northern pike. Future studies using more encompassing concentrations (*i.e*. lower and higher concentrations than those used here) are recommended to rule out false positives in all three species and life stages.

After swim-up, the LOAEC and NOAEC was determined for each species based on the most sensitive endpoints and only those two concentrations were continued until sexual differentiation. During this time frame, only lake trout fork length was significantly negatively affected at both the LOAEC and NOAEC concentrations.

2.6.3 ***Histopathology***

Once taken up into the body, Ag NPs may exert effects on cell membranes, ATP production, DNA replication, and gene expression (Sharma et al., 2014). This is supported by studies that have detected increased catalase activity in organisms exposed to Ag NPs, which is associated with ROS production (Choi et al., 2010; Shaw and Handy 2011). Edema is also a common outcome and is thought to be caused by osmoregulatory disruption, which was a commonly

observed malformation in the species studied here, particularly in the gills of larval fish (Wu et al., 2010).

The gills of fish are an active site of gaseous exchange, metabolite excretion and various other physiologically important processes (Shaw and Handy 2011; Chidambaram et al., 2014). Moreover, due to their external position, gills are frequently found to be a site of injury for aqueous contaminants (Shaw and Handy 2011; Chidambaram et al., 2014). There are two types of gill injuries: those that are initiated as a defense (hyperplasia of the filaments and epithelium, edema of lamellae) and those that result from direct injury (necrosis and shedding of the gill epithelium) (Chidambaram et al., 2014). Many metals are known to affect gill architecture because they are a primary organ for ion uptake and regulation and various studies have indicated that gills are also a main target of metal ion containing nanoparticles (Shaw and Handy 2011; Chidambaram et al., 2014; Johari et al., 2015; Ostasezwska et al., 2016). However, depending on size, some NPs may be too large to be taken up through the ion transporters and instead cause damage directly to membranes (Shaw and Handy 2011).

In this study, length to width ratios of secondary gill lamellae were not significantly different ($p=0.063$ and 0.061 for lake and rainbow trout, respectively), but there was a decreasing trend in the data. These non-significant results may be attributable to the immature nature of the gills considering the point in development when sampling took place. Regardless, any slight decrease may hold biological significance, especially in larvae and fry that are still developing. Therefore, further studies are required to determine if this change results in significant effects later in life, when the gills have fully matured, and development is complete. A decrease in length to width ratio signifies either a shortening or a thickening of the secondary lamellae, both of which are commonly used indicators of gill toxicity due to the decrease in respiratory ability

and ion exchange that both of these pathologies cause (Chidambaram et al., 2014; Johari et al., 2015). In a companion study of this project, which investigated proteomic and metabolomic outcomes of Ag NP exposures in rainbow trout and white sturgeon (*Acipenser transmontanus*), preliminary results suggest that exposure leads to disruption in pathways associated with gaseous exchange, including response to oxygen levels, as well as blood coagulation and platelet activation (Alcaraz et al., personal communication). Nanoparticles are known to inhibit ion transport, which is likely to lead to osmotic imbalance and instances of edema, whereas non-specific irritation is likely to cause hypertrophy of the epithelium resulting in fusion of secondary lamellae, edema, and increased mucous generation, which was found to occur at 96-h sublethal concentrations of 0.1 mg/L in Siberian sturgeon (*Acipenser baerii*) although this concentration is much higher than those used here (Ostaszewska et al., 2016). Congestion of lamellar blood vessels was also observed in this study, which may signify damage to pillar cells, which give structure to the lamellae (Ostaszewska et al., 2016). Moreover, hyaline degeneration, which is the storage of peptides from degraded cells, is known to be a distinct symptom of damage (Ostaszewska et al., 2016). Unfortunately, any adverse responses on the gills may lead to systemic effects due to the importance of the organs in homeostasis and the outcomes on whole-body circulation of metabolites and ions.

The liver is a major target organ and sequesters many environmental contaminants, including metals, and is the main organ for intermediary metabolism and energy storage (Scown et al., 2010; Chen et al., 2011; Shaw and Handy 2011; Kwok et al., 2012; Al-Bairuty et al., 2013; Griffitt et al., 2013). Furthermore, the liver also aids in recycling of endogenous materials from damaged cells and handles a large load in cases of systemic insults (Al-Bairuty et al., 2013). HSI and HVI were used to determine any changes in the liver in terms of its relative size compared to

the body (HSI) and the relative size of cells (HVI) between control and treatment groups. Therefore, an increase in HSI signifies an increase in the total size of the liver compared to the body, as was seen in lake trout at 3.00 nM, and an increase in HVI indicates smaller hepatocytes. Hence, if the HVI were to decrease, and fewer cells were counted within the confines of the predetermined field of view, such as in rainbow trout at 30.0 nM, it would indicate an enlargement of hepatocytes. However, rainbow trout exhibited an increased hepatosomatic index (HSI) in the 30.0 nM treatment and, when combining this information with the histopathological analysis of the liver, which showed a decreased hepatocyte volume index (HVI) and therefore a larger cell size, could implicate cellular impairments. In fact, multiple studies have found silver nanoparticles to be highly cytotoxic in rainbow trout by leading to decreased metabolic activity and membrane integrity, as well as increased oxidative stress and apoptosis (Choi et al., 2010; Farkas et al., 2010). A larger HSI could then be resultant from consistently larger hepatocytes due to swelling, whereas decreased metabolic activity could mean a decreased capability to utilize energy stores in the liver, which could result in excess lipids. This could implicate that HSI is not as reliable an endpoint in EC-exposed fish with disrupted metabolism as previously thought. In assessing the livers here, the increase in cellular size was shown to be due to increased instances of cloudy swelling, lipid accumulation, or vacuolization, all of which are indicators of toxicity and are indicative of non-specific necrotic lesions, which imply negative effects on liver function (Shaw and Handy 2011; Chen et al., 2011; Ostaszewska et al., 2016). Chen et al. (2011) also found that a 6-month exposure of titanium NPs in zebrafish caused an increased HSI and hypothesized that this was due to increased metabolism, resulting in liver injuries that led to increased lipidosis. Multiple studies have found that aqueous exposure to nanoparticles in various fish species results in increased liver size, likely owing to increased

metabolic load (Choi et al., 2010; Al-Bairuty et al., 2013). Although lipid accumulation is reversible, it can still lead to severe disruption of cellular function (Mumford et al., 2007). These impairments may have implications on metabolic function and capacity, particularly for rainbow trout which are known to traverse long distances to reach breeding grounds (Washington Fish and Wildlife Department (WFWD) 2008).

2.7 Conclusions

The use of NPs is increasing, and the present study outlines multiple effects of Ag NPs on early life stages of geographically relevant fishes in Canada. The main objectives of this study were to determine effects of long-term, multi-life stage exposures (starting at water-hardening and carrying through until approximate time of sexual differentiation) of varying concentrations of silver nanoparticles on the survival, growth, and development of three native Canadian species of commercial, recreational, and aboriginal concern. Our results not only suggest that the toxicity of Ag NPs is complex and species- and life stage-specific but also highlights the importance of conducting multi-life stage studies to fully encompass toxic effects in fish species, which is a sentiment that has been echoed elsewhere (Horie et al., 2017).

We found that rainbow trout, which represent a common laboratory species in toxicity tests for Canadian guidelines, seemed to be an adequate representative species for lake trout and northern pike as they were equally or more sensitive than the other species with regard to many of the most commonly-utilized ELS endpoints in toxicity testing. Results also show that there were life stage-specific differences in response to exposure to Ag NPs, with endpoints during the embryonic stage consistently the affected, and at lower concentrations, compared to larval and

fry stages. We also found evidence of impacts on growth and development below thresholds that lead to mortality. Certain endpoints, especially degree-days to hatch, were also consistently affected across species and life stages and seemed to be the most reliable when indicating exposure to Ag NPs.

Concurrent studies that aim to understand the specific molecular toxicity initiating events that drive adverse outcomes underlying exposure to Ag NPs are ongoing. In addition, future studies should build on the data presented here with further histological investigations to determine glycogen storage, mucous secretion, and goblet cells; immunohistochemistry (IHC) to better characterize irritation in the gill and liver and Masson's Trichome to determine fibrotic tissues in the gill and liver.

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Chapter 3 : **A multi life-stage comparison of fluoxetine toxicity on the early development of three Canadian fish species**

3.1 Preface

Chapter 3 expands on the work undertaken in Chapter 2 and focuses on the characterization of an aqueous fluoxetine hydrochloride exposure on the early life stages of rainbow trout, lake trout, and northern pike. This was completed in order to attempt to identify life stage- and species- dependent differences, particularly with regards to important – and often under-studied – species native to freshwater North American systems.

Author Contributions:

Dayna Schultz (University of Saskatchewan) designed and maintained the exposure studies, managed organisms and organized sampling events, generated and analyzed all data, and drafted the manuscript.

Song Tang (University of Saskatchewan; National Institute of Environmental Health, Chinese Center for Disease Control and Prevention) helped with experimental design, maintenance and chemical analysis of target analytes (Ag NPs)

Christie Miller (University of Lethbridge) assisted with managing exposure systems/organisms, and supported sampling events

Danielle Gagnon (University of Saskatchewan) assisted with the analysis of histological samples

David Janz (University of Saskatchewan) provided guidance for the experimental design and manuscript, as well as comments and edits.

Markus Hecker (University of Saskatchewan) provided overall guidance, advised with regard to experimental design, and provided comments and edits, as well as research funding.

3.2 Abstract

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine hydrochloride (FLX), also known as Prozac®, are emerging contaminants (ECs) of concern due their continuous release into the environment, long half-lives, high biological activity, and binding affinity to highly conserved receptors in non-target organisms such as fish. However, surprisingly little data are available on the toxicity of FLX in fish, and the limited data available has been generated on standard laboratory models non-native to northern climates. Given the diversity among fishes, extrapolation engenders significant uncertainty to whether available data is predictive and protective of other ecologically relevant species, particularly fishes of commercial, recreational, and aboriginal (CRA species) importance. In the present study, we obtained and fertilized gametes from three CRA species (rainbow trout (*Oncorhynchus mykiss)*, lake trout (*Salvelinus namaycush),* and northern pike (*Esox lucius*)), which were then immediately exposed to nominal waterborne concentrations of 0.5, 2.0, 8.0, 30.0, 125, and 500 μg/L FLX until sexual differentiation. Exposures were conducted under continuous flow-through conditions and endpoints included developmental rate (degree-days to 50% life stage transition), mortality, cumulative survival, embryonic malformations, and histological effects in livers of larval fish. Results suggest that, while the larval stage appeared to be the most sensitive, there were endpoint- and clear species-specific differences in responses. Fork length appeared to be the most sensitive endpoint for rainbow trout, with embryonic and larval lowest observable adverse effects concentrations (LOAECs) at 2.0 and 0.5 μg/L, respectively. Lake trout depicted a significant hormetic response for fork length with a significant decrease at 125 μg/L during the larval stage, as well as an increase in degree-days to swim-up at 0.5 μg/L. Northern pike only showed significant effects in degree-days to hatch and swim-up, where embryonic and larval

impacts occurred at 125 and 500 μg/L. Moreover, both rainbow trout and lake trout demonstrated significant mortalities at 125 and 500 μg/L. Further analysis revealed a significant decrease in hepatosomatic index (HSI) in lake trout fry, whereas histological analysis found increased hepatocyte volume index (HVI) in northern pike fry. To conclude, rainbow trout, which are a standard laboratory species in toxicity tests for Canadian water quality guidelines, was the most sensitive species at relevant concentrations of FLX, and therefore, seem to be an adequate representative species for lake trout and northern pike. Overall, this work will aid in the development of more appropriate environmental risk assessment strategies for native fishes to ECs of concern.

Keywords: Emerging Contaminants, Developmental Toxicity, Prozac, Selective Serotonin Reuptake Inhibitor (SSRI)

3.3 Introduction

Fluoxetine hydrochloride (FLX), commercially marketed as the pharmaceutical Prozac®™, is a selective serotonin reuptake inhibitor (SSRI) that is most notably used to treat psychological disorders in humans, such as depression, but may also be used to treat other neurological dysfunctions including compulsive behaviour and personality disorders (Brooks et al., 2003; Kreke and Dietrich 2008). FLX induces effects by inhibiting serotonin reuptake, a process that is conserved across other vertebrates, such as fish, as well as invertebrates, although the role and mode of action in many species is not known and may behave differently than in humans (Kreke and Dietrich 2008). In the past decades, the number of antidepressant prescriptions in Canada has been steadily increasing and, as of 2014, Canadians ingested approximately 3 million doses of antidepressants per day (Organization for Economic Co-operation and Development (OECD) 2017). FLX is prescribed as an oral medication and, after ingestion, is metabolized into the active metabolite norfluoxetine (norFLX), excreted in the urine, and eventually enters domestic wastewater (Kreke and Dietrich 2008). However, while fish are also thought to transform FLX into norFLX, one study reported that norFLX may not be the predominant biotransformation product in fish, which further complicates the present theories on the toxicokinetics and toxicodynamics of FLX in fish (Kreke and Dietrich 2008; Smith et al., 2010). More concerning is that the release of many pharmaceuticals into the environment is currently not actively regulated by Canadian, USA, or European governments, which limits the pressure to develop effective and affordable treatment technologies (Canadian Council of Ministers of the Environment (CCME) 2006; Jones-Lepp and Stevens 2007; Munoz et al 2008; Alvarez-Munoz et al., 2015; Kleywegt et al., 2019). Additionally, while there is a host of toxicity data available for humans and mammals, there is a significant lack of data for aquatic species.

However, detectable concentrations of FLX are being quantified in wastewater treatment plant effluents and are present in many surface waters (Kreke and Dietrich 2008; Christensen et al., 2009; Bringolf et al., 2010). One study found that the concentration of FLX in effluent being released into a North Carolina (USA) creek reached 0.12 μg/L (Bringolf et al., 2010) whereas a Spanish study found environmental concentrations of 0.40 – 0.93 μg/L, with sewage treatment effluent reaching as high as 1.14 μg/L (Christensen et al., 2009). Interestingly, a study by Kreke and Dietrich (2008), which reviewed the literature on FLX in the environment and fish, found that, while the concentration of FLX in the aquatic effluents and receiving waters was 99 and 46 ng/L, respectively, the concentrations of norFLX in influents was only 9.9 ng/L. One study that was based on environmental concentrations in the United Kingdom identified FLX as one of the pharmaceuticals of greatest concern to various aquatic organisms; it was ranked second based on a comparison of median effect and river concentrations (Donnachie et al., 2016). With increasing numbers of studies quantifying environmental concentrations paired with increases in the use of neuromodulators, there is an urgent need to understand the toxicological effects of FLX in fish species.

Once in the aquatic environment, the lipophilic FLX metabolite can be taken up into fish, mainly via the gills, and lead to physiological and behavioural alterations, affect endocrine and reproductive parameters, and cause increased incidences of malformations in early life stages (Foran et al., 2004; Kreke and Dietrich 2008; Corcoran et al., 2010; Weis 2014). The degree of absorption across the chorion needs further elucidation, as well as the effects that FLX might elicit on fish, amphibians and reptiles as compared to mammals. However, due to the highly conserved nature of the neuro-endocrine system, effects in mammals have previously been expected to be relevant for all vertebrates, although some evidence has arisen that found that

non-mammalian species may have slightly differing serotonergic systems, which could result in certain effects not observed in mammals (Kreke and Dietrich 2008; Corcoran et al., 2010). Corcoran et al. (2010) reviewed results that suggested FLX exposure can lead to decreased feeding rates and growth in fathead minnows (*Pimephales promelas*) at concentrations ranging from 51.0 to 170.0 µg/L for different FLX enantiomers. However, these concentrations are much higher than most of those found in the environment. Moreover, Foran et al. (2004) determined 48 LC_{50} values for invertebrates, fathead minnows and Japanese medaka (*Oryzias latipes*), at 230 µg/L, 710 µg/L and 8,900 µg/L, respectively. Furthermore, concentrations of FLX of > 200 µg/L induced skeletal deformities, muscular tail flexure, and facial malformations in the African clawed frog (*Xenopus laevis*), which suggests that FLX has teratogenic potential in amphibians and possibly other aquatic species. There is then an obvious lack of data available that adequately elucidates the adverse outcomes of FLX on the early development of fishes upon fertilization, effects on non-standard laboratory species (especially fishes native to North America), and potential for teratogenic and adverse effects at low concentrations and across multiple life stages. Research in this area is particularly relevant considering the likely steadily increasing environmental concentrations, specifically in regions with inadequate wastewater treatment such as those found in many Canadian municipalities.

In Canada, approximately 1,200 species of freshwater fish are present, which have diverse life history traits and ecosystem functions (Kenchington et al., 2013). Due to this biodiversity, CRA fish and fisheries are integral components of the ecosystem that aid in supporting the economy and other biota (Kenchington et al., 2013). Many CRA species, unlike commonly used laboratory species, frequently demonstrate long lives, slow growth, and slow regeneration times exposures to ECs during early development have the potential to be

detrimental to populations. Early life stages (ELS), which include the periods from fertilization until sexual differentiation, of many aquatic species have been shown to be the most sensitive to contaminant exposures, and several studies suggest that even low concentrations can have immediate or latent effects on individuals and populations (Embry et al., 2010; Weis 2014). The ELS of fishes, similarly to humans, include stages of organogenesis, organ migration, body growth, and differentiation of the gonads. Data regarding the fate and distribution of FLX in the aquatic environment, as well as the toxicokinetic and toxicodynamic processes in fish are lacking, which is likely due to the complexity of receiving environments on pharmaceutical contaminants as well as the intricacy and relatively unknown nature of the endocrine system of vertebrates. Also, owing to the uniqueness of each receiving environment, exposure characteristics are not easily generalizable. Therefore, considering the potentially detrimental implications that FLX may impart on receiving environments and the abundance of biodiversity they support, further research is necessary.

In the current study, three CRA species were used to assess the potential effects of FLX on the earliest developmental stages (embryo, larval, fry), and also to elucidate differences between species and life stages. Very little is known about the consequences of FLX on ELS of native Canadian fishes, especially immediately after fertilization, when an initial exposure prior to or upon water hardening may lead to adverse responses. This study will help to elucidate the toxic effects and potential hazards that FLX may pose and, in turn, will add valuable knowledge to the scientific community that will potentially influence future chemical safety and wastewater regulations in order to more objectively and reliably protect the aquatic environment and relevant biota.

3.4 Materials and Methods

3.4.1 ***Chemicals***

Stock solutions of FLX (as fluoxetine hydrochloride) were prepared from >98% pure standards (Alfa Aesar, Wardhill, MA, USA).

3.4.2 ***Fish Collection and Egg Fertilization***

Spawning lake trout and northern pike were collected in October 2015 and May 2016, respectively. Lake trout were collected from Lac la Plonge (55°08′N 107°20′W; n = 3 males and 1 female) whereas northern pike were collected from Lac la Ronge (55°10′N 105°00′W; n = 4 males and 2 females). Both Lac la Ronge and Lac la Plonge are clean, large, glacial lakes located in Northern Saskatchewan. Ripe males and females were obtained using gill nets and placed in a holding tank for < 2 hours before gamete collection. Weights and fork lengths were then measured, and eggs or milt collected by gently applying pressure to the abdomen. Male fish were released after milt collection while female fish were either released or euthanized by blunt-force trauma for further tissue sampling. Rainbow trout gametes from three male and three female trout were obtained from Troutlodge (Sumner, WA, USA).

All materials used during egg fertilizations were sterilized with either 75% ethanol or 0.000075% Proviodine® (povidone-iodine; Rougier Pharma, Mirable, QC, Canada) solution. Milt from males and eggs from females were first pooled before fertilization. For rainbow trout and lake trout, eggs and milt were mixed for approximately 2 min before activation with water (Aquatic Toxicology Research Facility water and site water, respectively), whereas northern pike were gently stirred with a slurry of bentonite clay to bind and minimize mucous. For lake trout

and northern pike, fertilized eggs were immediately transferred to either plastic bags or glass mason jars containing 1 L of exposure solution and oxygenated, then placed in coolers for transportation. For all species, transfer to exposure solutions occurred within an hour of fertilization. Embryos were allowed to water harden in exposure solutions for at least 12 hours before being transported to the University of Saskatchewan so as to ensure the initial exposure upon water hardening and reduce susceptibility to mechanical damage immediately after fertilization. For rainbow trout, embryos were transferred directly to 10-L exposure tanks. Embryos were transferred randomly to exposure solutions, with each replicate containing approximately 120 embryos (rainbow trout) or 80 embryos (lake trout, northern pike).

3.4.3 ***Experimental Setup***

All animal use was approved by the University of Saskatchewan's Animal Research Ethics Board (AUP#: 20140079) and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Exposures were conducted in the Aquatic Toxicology Research Facility (ATRF) at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). Test organisms were exposed to 6 concentrations of aqueous FLX (0.50, 2.00, 8.00, 32.0, 125, and 500 μg/L), which were diluted 1:10 000 with ATRF water, as well as controls. Each treatment contained three replicate tanks containing two egg chambers (cleaned and presoaked PVC pipe with a mesh bottom) to separate the different life stages and allow for easy inspection. Tanks were held in a water bath that was equipped with a temperature regulator and thermometer to control temperatures amongst treatments over the duration of the experiment.

3.4.4 ***Incubations***

Upon arrival at the ATRF, rainbow trout embryos were transferred directly to flow-through exposure tanks whereas lake trout and northern pike were transferred into aerated 1-L glass jars and later transferred to flow-through tanks upon reaching the eyed stage. ATRF water quality was measured daily for dissolved oxygen, pH, and conductivity, and bi-weekly for ammonia, alkalinity, and nitrates using a YSI Quatro Professional Plus multiparameter field cable (Yellow Springs, USA) or testing kits (LaMotte, Chestertown, USA), respectively. Further water quality can be found in the supplementary materials (Appendix A). Temperatures mimicked environmental conditions so that temperatures for each species were between 12 and 14ºC for rainbow trout, 10 and 12ºC for lake trout, or, for northern pike, started at 10 and gradually increased to a maximum of 15ºC. Embryos were examined daily for fungal infections, morbidities, mortalities, and unfertilized and undeveloped/dead embryos, and dead individuals were removed. After 48 hours, fertilization success was determined for all replicates (total dead embryos at 48 h/ total number of eggs · 100). Fertilization success was greater than 90% for lake trout, 75% for rainbow trout, and 98% for northern pike. Numbers of fish transitioning to the next life stage (hatch, swim-up) per day were recorded and individuals were transferred either from the embryo cup to the larval cup (after hatch) or from the larval cup into the tank (after swim-up) in order to accurately count and determine life stage-specific endpoints. When 50% of organisms within a tank transitioned, the median time to transition was determined. After all viable organisms had transitioned to the next life stage, a sub-portion of the population was euthanized using an overdose of buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate) and immersed in buffered formalin for 24 – 48 h before being transferred into 70% ethanol for

storage until histological examination of liver (larval stage only) occurred. During the exposure, culling was carried out as necessary in order to reduce crowding.

Apical endpoints for the embryonic stage included cumulative degree-days to 50% hatch, mortality, and fork length across all species, as well as malformations upon hatch (presence/absence of skeletal curvatures, edema, hemorrhage, craniofacial malformations) assessment in rainbow trout and lake trout only. Apical endpoints for larvae included cumulative degree-days to 50% swim-up, mortality, and fork length. After swim-up, the lowest observable adverse effect concentration (LOAEC) and no observable adverse effect concentration (NOAEC) were determined for available treatments, and only LOAEC and NOAEC concentrations were carried out for the remainder of the exposure. During the fry stage, fish were fed ad libitum with commercial start-up feed (rainbow trout and lake trout) or brine shrimp (northern pike) at least twice per day (Environment Canada (EC) 1998). After the estimated time to sexual differentiation occurred, the remaining LOAEC and NOAEC concentrations were terminated and apical endpoints, including morality, and fork length, where applicable, were measured. Hepatosomatic index (HSI; Equation 4) was calculated where possible (lake and rainbow trout fry) and omitted for northern pike as body size was too small to perform dissections.

$$HSI = \frac{Weight_{liver}}{Weight_{total}} \quad (4)$$

Assessments of deformities, growth, and development in fry were conducted as outlined in the EC (1998) report on toxicity tests in early life stage salmonids, whereby embryo/alevin/fry mortality of up to 40% by the time of 50% swim-up is acceptable before the test becomes non-viable.

3.4.5 ***Chemical Analysis***

Exposure solutions were sampled at the beginning of the exposures and measured by liquid chromatography-mass spectrometry (LC-MS; Table 3.1). Stock solutions of fluoxetine and its deuterated counterpart (fluoxetine-d5; Cayman Chemicals, Ann Arbour, MI, USA) were prepared in HPLC grade methanol (Thermo Fisher Scientific, Waltham, MA, USA) at 100 mg/L. A 7-point calibration curve ranging from 0.5 – 500 µg/L and spiked with 50 µg/L fluoxetine-d5 was used for quantification by isotope dilution (linearity > 0.999 for all analyses). All exposure solutions were sub-sampled (1 mL) directly into LC vials and spiked with fluoxetine-d5 at a target concentration of 50 µg/L. Fluoxetine concentrations were confirmed using a Vanquish UHPLC and Q-ExactiveTM HF Quadrupole-OrbitrapTM mass spectrometer (Thermo Fisher Scientific). LC separation was achieved with a Kinetex 1.7µm Biphenyl LC column (100 x 2.1 mm) (Phenomenex, Torrance, CA, USA) using an isocratic elution of 40% H_2O: 60% acetonitrile (Thermo Fisher Scientific) at a flow rate of 0.2 mL/min and column temperature of 40^{o}C. Fluoxetine and fluoxetine-d5 had a retention time of 1.5 min. Samples were ionized by positive mode heated electrospray ionization (HESI) with the following source parameters: sheath gas flow = 20; aux gas flow = 5; sweep gas flow = 1; aux gas heater = 300^{o}C; spray voltage = 3.5 kV; S-lens RF = 60; capillary temperature = 350^{o}C. A targeted-SIM and PRM (collision energy = 20) method at 60,000 resolution, AGC target = 1x106, and max injection time = 100 ms was used to monitor [M+H]+ precursor and product ions of fluoxetine (m/z 310.141 → 148.112) and fluoxetine-d5 (m/z 315.173 → 153.143). Precursor and product ions were used for quantification and confirmation, respectively.

3.4.6 ***Histopathology***

Whole-mount fish were processed and embedded in accordance with the protocol in place at the Western College of Veterinary Medicine (Saskatoon, SK). Samples were sagittally sectioned to 5 – 7μm thickness and stained following the routine hematoxylin and eosin protocol in place at the Toxicology Centre (Saskatoon, SK). Evaluation of histopathologies were completed using an Olympus model BX41 microscope (Olympus America, Melville, NY, USA) with Infinity Capture and Infinity Analyze Software (Ver 6.5.4: Lumenera Corporation, Ottawa, ON, Canada). Quantitative analysis of the liver in rainbow trout ($n_{control}$=10, $n_{treatment}$=10), lake trout ($n_{control}$=9, $n_{treatment}$=6), and northern pike ($n_{control}$=6, $n_{treatment}$=8) was measured using the hepatocyte volume index (HVI) by counting the number of cells within a 100 μm^2 area of three random views (40X magnification) (Palace et al., 2002). HVI analysis was used as an indicator of the cellular size of liver parenchyma so that a relatively lower HVI indicates an increase in cellular size.

3.4.7 ***Statistical Analysis***

Data were tested for normality (Shapiro-Wilkes test) and equality of variance (Brown-Forsythe Test for Homogeneity of Variance) using GraphPad Prism 7 (San Diego, CA, USA). To determine significant differences between the controls and three or more treatment groups, a one-way analysis of variance (ANOVA) with post-hoc analysis (Dunnett's t-test) were performed for parametric data. Non-parametric data were analyzed using a Kruskal-Wallis H test with post-hoc analysis (Dunn's t-test). For fewer than three treatment comparisons, an unpaired T-Test was used in cases of parametric data or a Mann-Whitney test (Exact p-value) on nonparametric data. Data that passed parametric assumptions are presented as mean ± one standard error of the mean (SEM), and those that did not are presented as the median ± interquartile range (25^{th} percentile,

75th percentile). All statistical tests were 2-tailed and used an alpha value of 0.05. Due to sampling complications, for non-LOAEC and NOAEC concentrations, larval subsampling took place into the fry stage and therefore, these data were used for fry life stage data, although it does not extend as far as the LOAEC and NOAEC concentration data.

Survival data were generated by using count values obtained throughout the duration of the exposures and analyzed in Microsoft Excel®. Data from each life stage was combined and averaged across replicates in order to analyze cumulative survival of individual treatments at the end of the study and throughout. Replicates were then averaged. Survival analysis takes into consideration all fish used in the study, including those that are culled, sampled, or that died for unexpected reasons throughout the duration of the study. Random, large numbers of deaths (*i.e.* greater than 10% of individuals seeded per life stage per replicate; not attributable to water quality) were uncommon throughout the exposures and used to identify potentially confounding factors. These data were censored in most cases except when they occurred during the life stage transitioning window, as this window is known to be a time of naturally high mortalities or when they occurred with other indicators of morbidity, such as listlessness or moribundity (Vardy et al., 2014).

To determine the percent surviving on a given day ($Survival_t$), the percent surviving on the previous day ($Survival_{t-1}$) was multiplied by the proportion of the total that remained alive on the day of interest (Equation 5). This was obtained by subtracting the sum of dead on the day of interest ($\sum Dead_t$) from the sum of surviving on the previous day ($\sum Alive_{t-1}$) and dividing by the sum of surviving on the previous day.

$$Survival_t = (Survival_{t-1})\ x\ \left(\frac{\sum Alive_{t-1} - \sum Dead_t}{\sum Alive_{t-1}}\right) \quad (5)$$

3.5 Results

3.5.1 ***Chemical Analysis***

Water samples were analyzed to determine the concentration of FLX in solution and the percent of target concentration (Table 3.1). Percent of target concentrations decreased from the highest concentrations to the lowest concentrations with the lowest two treatments exhibiting levels below detectable limits whereas 125 and 500 μg/L treatments were within close range to the target concentrations. ATRF facility water remained within allowable ranges (dissolved oxygen > 80%; pH 7.6 – 8.0; conductivity 220 μS/cm; ammonia < 0.02 μg/L) throughout the duration of exposures.

Table 3.1: Concentration of FLX in exposure solutions associated with nominal concentrations (μg/L). Measured concentrations are given as averages ± 1 standard deviation (μg/L), < 0.40 for less than level of detection (LOD), and percent of target concentration. Water samples were measured using LC-MS.

Nominal Concentration (μg/L)	Average Measured Concentration (μg/L)	Percent of Target Concentration (%)
0.00	< 0.40	-
0.50	< 0.40	-
2.00	< 0.40	-
8.00	1.59 ± 1.05	19.9
32.0	18.2 ± 8.9	57.0
125	119.6 ± 6.9	95.7
500	495.4 ± 68.9	99.1

3.5.2 ***Survival Analysis***

Each species varied in their cumulative survival dynamics upon continuous, long-term exposures to FLX (Figure 3.1). For lake trout, the time of life stage transition to hatch and swim-up corresponded to 32 – 48 days post fertilization (dpf) and 69 – 93 dpf, respectively (Figure 3.1A). Hatching and swim-up in rainbow trout occurred between 31 and 43 dpf, and between 42 and 64 dpf, respectively (Figure 3.1B), while northern pike hatched between 7 and 30 dpf and swam up between 13 and 25 dpf (Figure 3.1C).

Lake trout, rainbow trout, and northern pike survival all trended downwards within the transition windows, although the degree of mortality was dependent upon exposure concentration. Rainbow trout exhibited high survival until hatch and after swim-up (Figure 3.1B). However, fish in 125 and 500 μg/L treatment groups failed to survive past swim-up ($p < 0.0001$) whereas those in the lowest two concentrations showed a decreased survival, although only significant for the 0.50 μg/L group ($p < 0.05$). The 0.50, 125, and 500 μg/L groups were significantly different from control with 66.4 ± 12.6% survival in the 0.50 μg/L group versus 0% survival in the 125 and 500 μg/L groups. Alternately, northern pike exhibited a constant decline in survival, likely owing to the overlapping life-stage transition windows. Responses in survival to FLX exposure did not seem to exhibit any consistent trend, although exposure to 0.50 and 8.00 μg/L demonstrated a significant decrease in survival from controls (51.3 ± 4.14% and 49.9 ± 5.66% versus control survival of 73.3 ± 2.29%, respectively; $p < 0.005$). However, there were no significant differences in the survival of fish exposed to 2.00, 32.0, 125, and 500 μg/L FLX, with survival ranging from 60-70%.

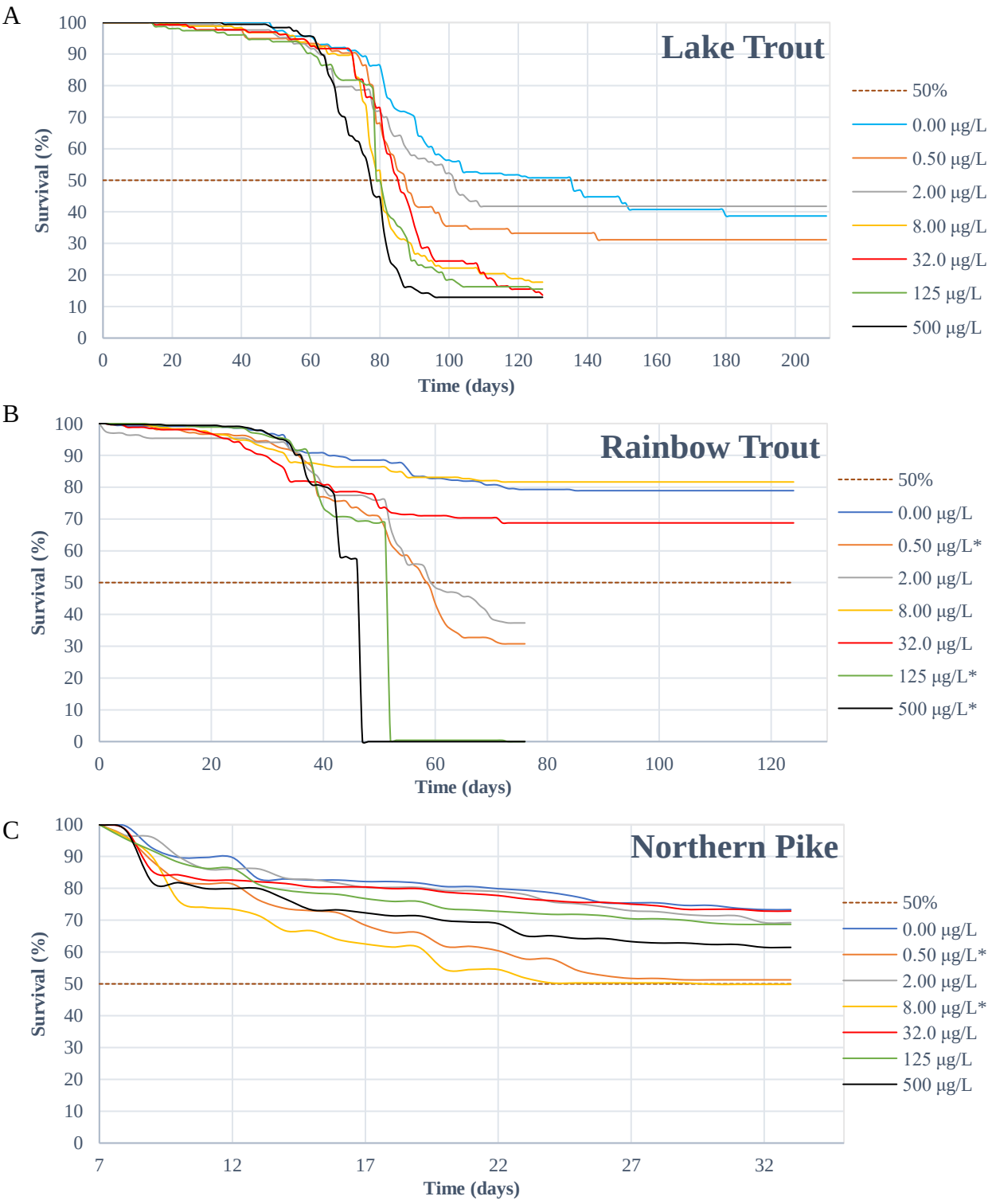


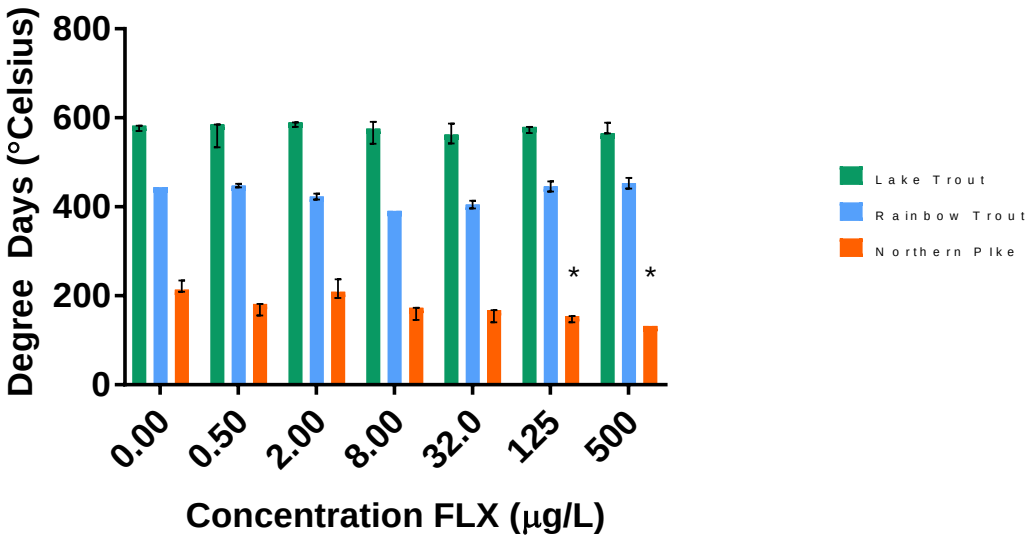
Figure 3.1: Survival analysis of lake trout (A), rainbow trout (B), and northern pike (C) from each treatment (replicates averaged) to give an overall treatment-specific survival curve for the

FLX toxicity test from fertilization until approximate sexual differentiation (lake and rainbow trout) and eyed until swim-up (northern pike). (*) indicate statistically significant differences from controls at the end of the study period ($p < 0.05$).

3.5.3 ***Apical Endpoints in Embryos***

Developmental time was only significantly impacted in northern pike and fork length at hatch was only affected in rainbow trout (Figure 2). Northern pike displayed a significant decrease in time to hatch when exposed to 125 μg/L with 155 degree-days (140, 157) and 500 μg/L with 132 degree-days (132, 132) compared to 214 degree-days (209, 234) in controls ($p < 0.05$). Conversely, rainbow trout were the only species to exhibit a significant decrease in fork length at hatch with measurements of 20.5 mm (18.5, 22.0) in controls versus 16.5 mm (14.0, 17.0), 17.0 mm (16.0, 18.8) and 16.8 mm (15.5, 19.3) in the 2.00 μg/L, 125 μg/L and 500 μg/L treatment groups ($p < 0.005$).

A



B

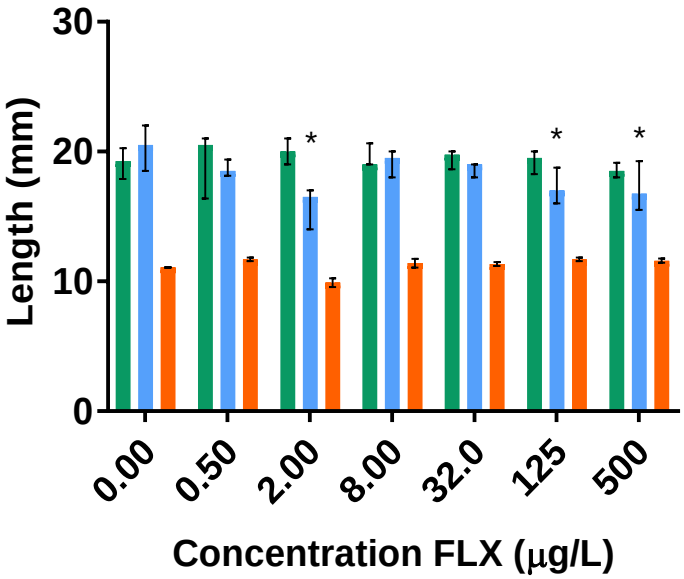


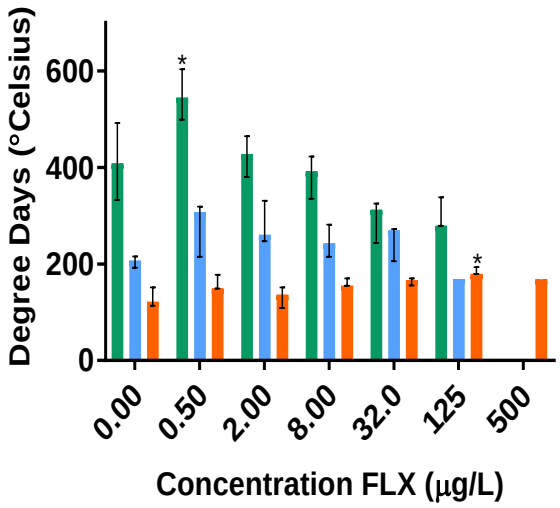
Figure 3.2: Effects of increasing concentrations of FLX from fertilization to hatch on lake trout (green), rainbow trout (blue), and northern pike (orange) embryos for (A) degree-days (°C) and (B) length (mm). Bars represent the mean +/- SEM or median and upper and lower limit of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

3.5.4 ***Apical Endpoints in Larvae***

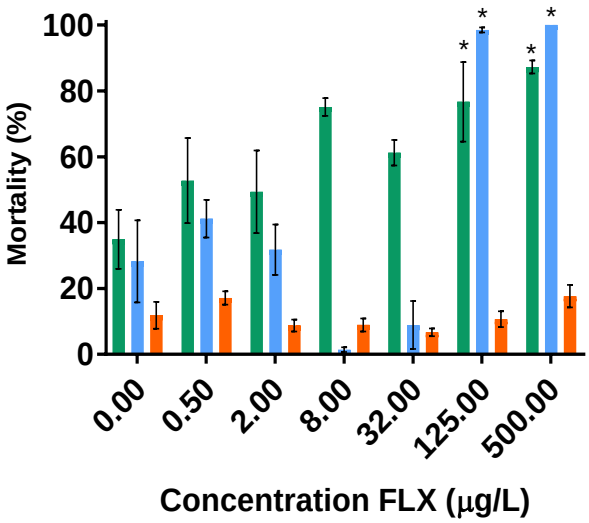
Significant effects were noted for developmental time in lake trout, as well as mortality and fork length for lake trout and rainbow trout (Figure 3.3). For lake trout, a significant increase in developmental time was noted after exposure to the lowest concentration of 0.50 μg/L (549 ± 30.3 degree-days) compared to controls (411 ± 46.0 degree-days; $p < 0.05$). Interestingly, a subsequent concentration-dependent downward trend was noted thereafter. Rainbow trout, however, depicted no obvious trend or significant effects for developmental time to swim-up, with degree days ranging from 169 to 269. Northern pike showed an increase in developmental time to swim-up where significance was noted in the 125 μg/L treatment group of 179 (179, 194) degree-days compared to control times of 122 (113, 151) degree-days ($p < 0.005$). Northern pike also appeared to be relatively unaffected in terms of mortality resulting from FLX exposures (all mortality was less than 20%) while both trout species showed a relatively higher sensitivity (Figure 3.3 B). In lake trout, a significant effect on mortality was observed in fish exposed to the highest two concentrations, 125 and 500 μg/L (76.7 ± 12.1% and 87.3 ± 1.99%, respectively), compared to controls (34.9 ± 8.94%; $p < 0.05$). Although only the highest two concentrations had mortalities that were statistically significant, there appeared to be a concentration-dependent response. Conversely, rainbow trout showed a response whereby the lowest two concentrations (0.50 and 2.00 μg/L) had mortality near control values (41.2 ± 5.70% and 31.8 ± 7.63%), the middle concentrations (8.00 and 32.0 μg/L) had very low mortality (1.42 ± 0.709% and 8.90 ± 7.27%), and the highest two concentrations (125 and 500 μg/L) resulted in significantly heightened mortality (98.5 ± 0.762% and 100 ± 0.00%) compared to controls (41.9 ± 13.3%; $p < 0.05$). With regards to size at swim-up, lake trout exhibited, similarly to degree-days, an apparent hormetic trend whereby the lowest concentration depicted a relative increase and subsequent

decrease, culminating in a significant decrease in fork length at 125 μg/L (26.2 ± 0.655 mm) compared to controls (30.6 ± 0.0622 mm; $p < 0.005$). Moreover, exposed individuals displayed a noticeably larger variation in size in exposure groups. In contrast, rainbow trout did not show a consistent concentration-dependent trend; significant effects only occurred at the two lowest concentrations of 0.50 and 2.00 μg/L, both of which had individuals with lengths of 28.0 mm (26.5, 30.5 and 26.0, 30.0, respectively) compared to control lengths of 31.0 mm (29.0, 32.4; $p < 0.0001$). Both mid-range treatments exhibited larvae similar in size to controls.

A



B



C

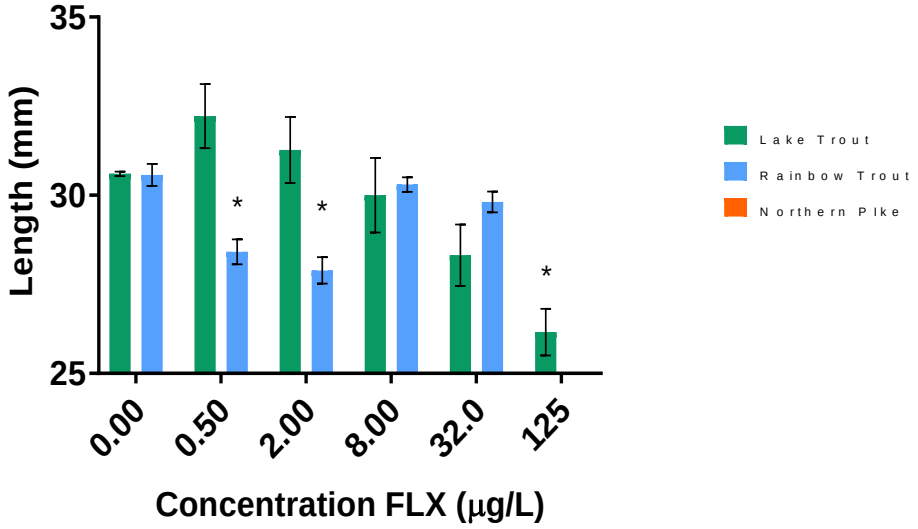


Figure 3.3: Effects of increasing concentrations of FLX from hatch to swim-up in lake trout (green) and rainbow trout (blue) larvae for (A) degree-days (°C) and (B) mortality, and (C) fork length (mm). Bars represent the mean +/- SEM or median and upper and lower limit of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

3.5.5 ***Apical Endpoints in Fry***

Percent mortality for rainbow trout displayed a significant increase for the 125 μg/L group, which resulted in 100% mortality in the highest two treatment groups for that species (Figure 3.4; $p < 0.05$). There were no significant effects in fork length of fry for any species. HSI was measured in lake and rainbow trout but omitted for northern pike due to their small size (Figure 3.5). Lake trout depicted a decrease in HSI from 1.64 ± 0.135 in controls to 1.54 ± 0.051 and 1.20 ± 0.088 in 0.50 and 2.00 μg/L treatments, respectively. However, only the 2.00 μg/L treatment was significantly affected ($p < 0.05$). Conversely, rainbow trout showed no significant effect for HSI with control values of 1.20 (0.881, 1.39) compared to 1.24 (1.05, 1.40) and 1.16 (0.972, 1.42) at 8.00 μg/L and 32.0 μg/L, respectively (Figure 3.5B).

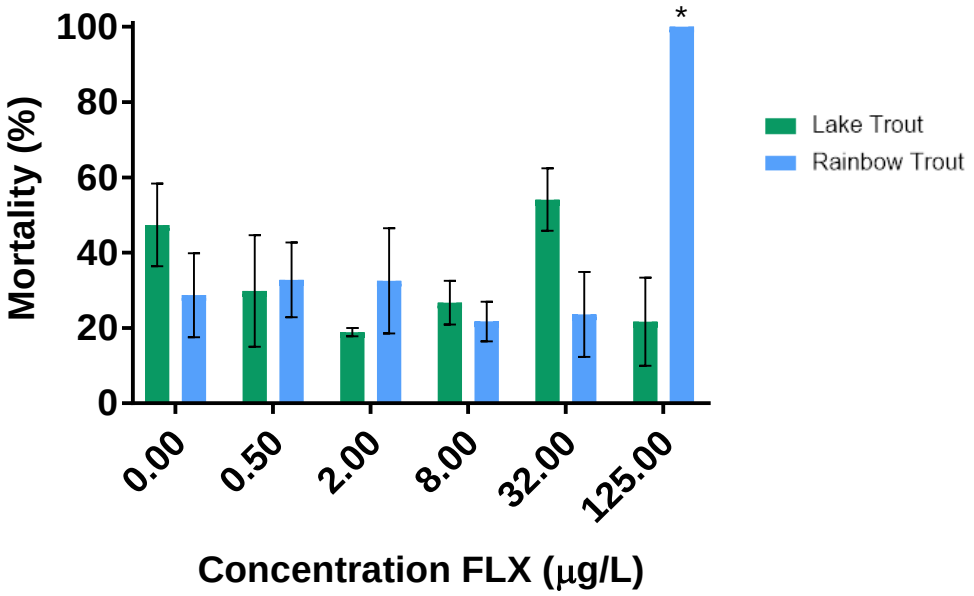


Figure 3.4: Effects of increasing concentrations of FLX from swim-up to take down of lake trout (green) and rainbow trout (blue) fry for mortality (%). Bars represent the mean +/- SEM or median and upper and lower limit of replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

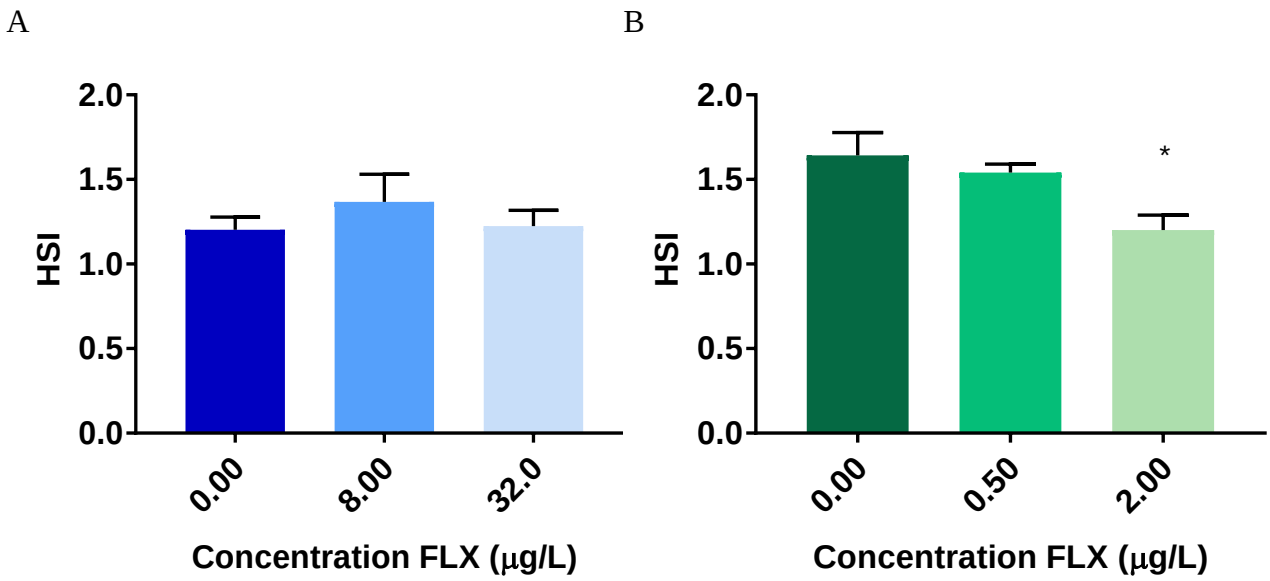


Figure 3.5: Hepatosomatic index (HSI) of rainbow trout (A) and lake trout (B) fry after exposure to the LOAEC concentrations of FLX. Bars represent the mean +/- SEM of 3 replicates. (*) indicates statistically significant differences from controls ($p < 0.05$).

3.5.6 ***Histopathology***

Exposure to FLX led to a significant decrease in hepatocyte size in northern pike, while no significant effects in either trout species were observed, and only minor alterations in histopathological analysis occurred. With the exception of HVI, histological examination of the livers of FLX-exposed fishes showed few alterations (Figure 3.6). Lake trout and rainbow trout showed no significant effect on HVI. In lake trout, control values of 33.2 ± 1.69 compared to 35.4 ± 1.71 for 125 μg/L, while rainbow trout control values of 37.9 ± 2.64 compared to 41.5 ±1.45 in 32.0 μg/L show an increasing trend. In contrast, northern pike had control values of 41.9 ± 2.40 compared to 55.0 ± 2.12 in the 125 μg/L treatment group ($p < 0.01$; Figure 3.6C). These data suggest that the hepatocytes in exposed northern pike were smaller than those in control organisms.

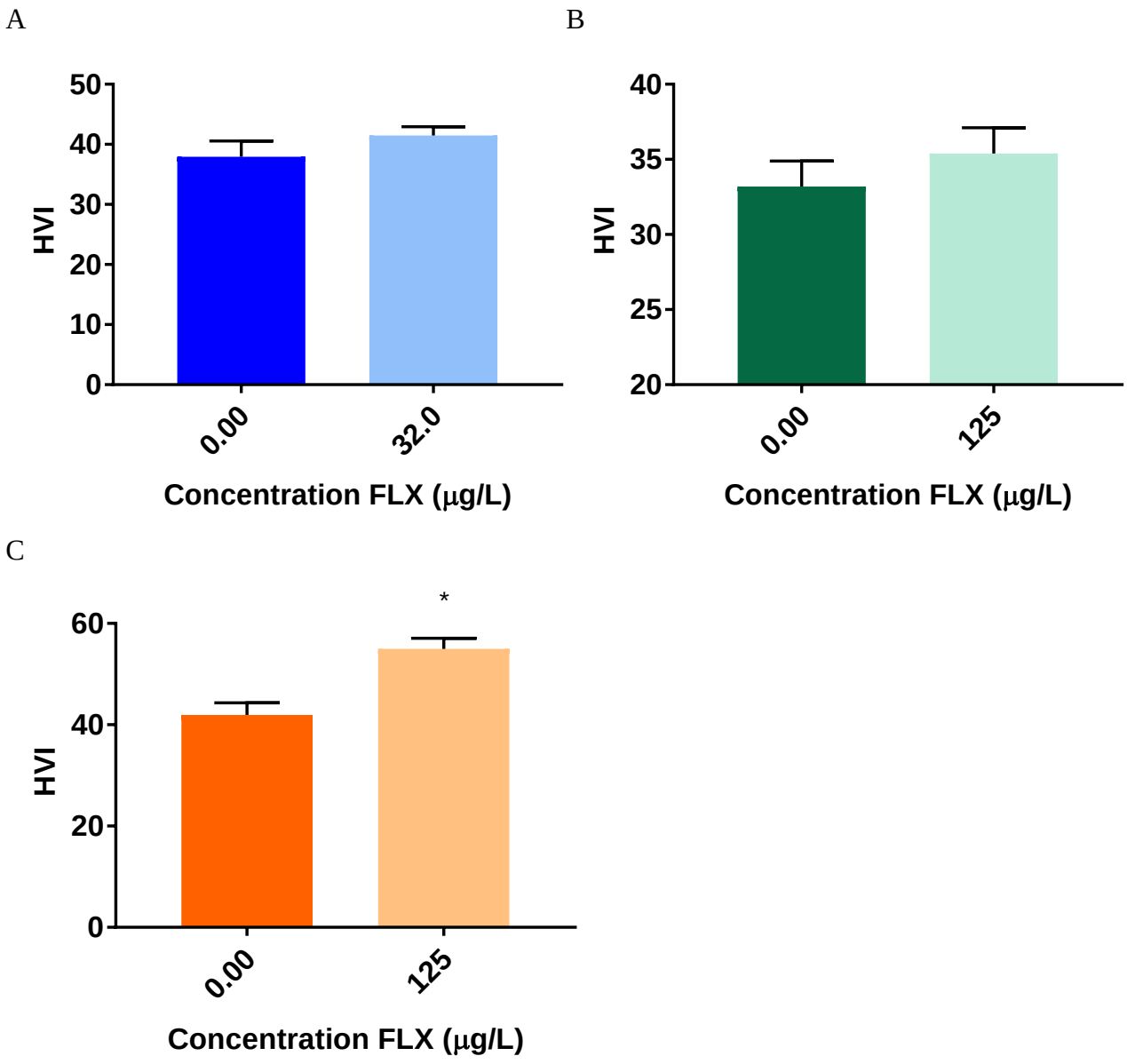


Figure 3.6: Hepatocyte volume index (HVI), as an indicator of cell size, in rainbow trout (A), lake trout (B), and northern pike (C) fry after exposure to highest concentrations of FLX. Bars represent the mean +/- SEM for replicates. (*) indicates significance compared to controls ($p < 0.01$).

Histological examination of the livers of FLX-exposed fishes showed few alterations (Figure 3.7). While lake trout and rainbow trout livers appeared unremarkable, northern pike displayed an obvious decrease in hepatocyte size.

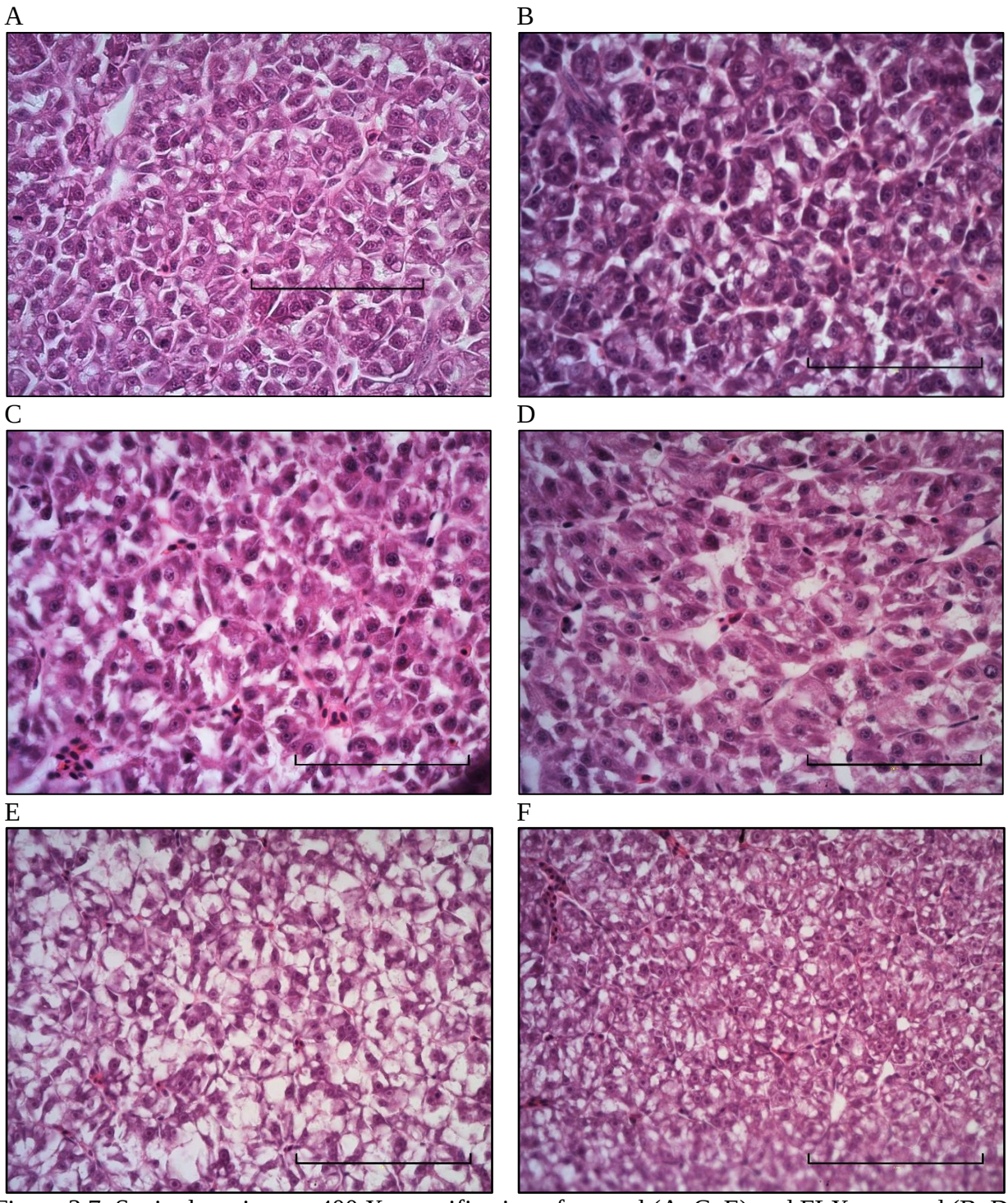


Figure 3.7: Sagittal sections at 400 X magnification of control (A, C, E) and FLX-exposed (B, D, F) livers of rainbow trout (top), lake trout (middle) and northern pike (bottom) fry (125 μg/L). Control livers demonstrate normal parenchymal architecture. (B) demonstrates decreased hepatocyte size. Bar = 100 μm.

3.6 Discussion

3.6.1 ***Survival Analysis***

Unfortunately, very little data exist to outline the mechanism of incorporation of FLX into the developing embryos of fishes, although studies suggest that uptake across the chorion is negligible (Kreke and Dietrich 2008). In larvae and fry, FLX was shown to be taken up across the gills, where it may sequester (Kreke and Dietrich 2008). These concepts were supported in the survival analysis conducted in this study, where survival during the embryonic stage was not affected by FLX in any of the species investigated, while there were marked decreases in survival that corresponded with the onset of gill development in the late larval/early fry stage in rainbow trout. However, impacts in lake trout occurred before the onset of gill development and were therefore likely mediated through the epithelial surface. Rainbow trout depicted a peculiar response whereby the highest and lowest two concentrations resulted in high mortality, but the mid-range concentrations did not. Some studies have also noted a biphasic response towards FLX as well as other psychiatric drugs (de Farias et al., 2019). This could Moreover, while northern pike did display some significant impacts on mortality, this seemed to be due to random effects at these life stages, although values are only provided for days 7 to 33 dpf.

The results here demonstrated that, while individual life stage analysis seems adequate to identify overt toxicity at high concentrations (*i.e.* 125 and 500 μg/L), it may be ill-suited at accurately predicting long-term toxicity in low concentrations. This is somewhat concerning considering 0.50 μg/L was the most environmentally relevant concentration used. Early life stage fish are generally confined to the vicinity of the spawning grounds of their parents until the fall when ice begins to form, after which they leave the shallows for deeper water. In the present study, survival analysis was a useful technique to employ and use as a comparison against

individual life stage studies. It was especially useful to characterize long-term, low-concentration effects of FLX throughout several sensitive life stages that individual life stage studies may inadvertently overlook. However, it also requires a very precise control of parameters, including temperature and water quality, and provides an extended length of time for complications to occur, which makes it a logistically challenging approach to effectively utilize.

3.6.2 ***Apical Endpoints***

While results from the survival analysis indicated negligible diffusion of FLX across the chorion, results from the apical endpoints in embryos suggest that there was at least some uptake either across the chorion or during water hardening that led to decreases in embryonic growth indices in rainbow trout and decreased developmental time to hatch in pike. Also, while not significant, lake trout displayed an increasing trend in malformations as concentration increased (Appendix C). Multiple studies have found that the offspring of Japanese medaka exposed to FLX displayed incomplete development, edema, and spinal curvatures at concentrations ranging from 0.1 to 5.0 μg/L and that developmental abnormalities were approximately 5 times more frequent in FLX-exposed embryos (Brooks et al., 2003; Foran et al., 2004). However, it seemed (though it was not explicitly stated) that both studies removed the embryos from the exposure concentrations after water hardening and placed them in clean water. Therefore, by combining the results from those studies and the present study, it seems that each study reported some incidence of developmental abnormalities. And, while the mechanism of exposure was not elucidated in the aforementioned studies, effects on developing embryos may be due to the initial uptake of FLX upon water hardening and not due to impacts on parental gametes or maternal transfer. However, a recent study by Martinez et al. (2019) uncovered evidence that suggests that

maternal exposure to FLX may affect the stress axis response in offspring, which resulted in dysregulation of epigenetic transcripts. While the present study did not see any clear signs of teratogenicity, we did find stunted growth, which could be attributed in part to minor skeletal malformations in combination with stunted growth and development. In a parallel study, Alcaraz et al. (in progress) found transcriptomic evidence that skeletal development pathways were indeed being affected after exposure of early life stage rainbow trout to FLX at 125 μg/L. However, it is important to note that the present study only screened for malformations whereas multiple other studies included a more thorough assessment of developmental endpoints that were not able to be measured here (*i.e.* behavioural responses, specific organ toxicity, skeletal malformations, etc.). Hence, embryos with minor malformations were likely inadvertently missed in the present study.

Various studies have quantified the adverse outcomes of FLX on growth and mortality endpoints, although most have analyzed effects in adult fish (Foran et al., 2004; Gaworecki and Klain 2008; Corcoran et al., 2010; Mennigen et al., 2010; de Farias et al., 2019). However, one study found that 168-h acute toxicity in embryonic zebrafish (*Danio rerio)* occurred at 1.18 mg/L, which is much higher than environmental concentrations as well as those used here (de Farias et al., 2019). In another study conducted in Brazil, exposures to a reference value, which was based on concentrations in a local river of 0.99 μg/L, as well as incrementally increasing concentrations, resulted in earlier hatching and decreased survival in zebrafish, which is similar to the developmental effects noted in northern pike but at a much higher concentration (Kalichak et al., 2016). Moreover, the authors found that fish exposed to higher concentrations (99 μg/L) had decreased heart rate and were smaller in size compared to controls, which, size-wise, was also noted in rainbow trout exposed here, and suggests that the embryos and larvae are somehow

internalizing the drugs (Kalichak et al., 2016). However, other findings are contradictory. A study in Western mosquitofish (*Gambusia affinis*) exposed to 0.05 to 5 μg/L waterborne FLX from hatch until 91 days post hatch did not show any effects on survival, growth, or sex ratio although it did lead to increased lethargy at > 0.5 μg/L (Henry and Black 2008). However, Foran et al. (2004) found condition factor to be unaffected in adult-treated Japanese medaka. This further illuminates the complexity of the toxicological effects of this pharmaceutical and the presence of species- and life stage- specific differences (Henry and Black 2008). Together, these results imply that early life stage fish are much more vulnerable to exposures to FLX than adults or juveniles.

There is abundant evidence that supports the crucial role that serotonin plays in embryo development in (Kreke and Dietrich 2008; Ulhaq and Kishida 2018; Martinez et al., 2019). These findings could further help to explain the developmental effects noted here. However, a major constraint to determining effects on fishes is the toxicokinetics of FLX. While Margiotta-Casaluci et al. (2014) found that norFLX is a common metabolite in fathead minnows, Smith et al. (2010) found that FLX loss was greater than norFLX production in rainbow trout, goldfish (*Carassius auratus*), zebrafish, and Mummichog killifish (*Fundulus heteroclitus*), suggesting that norFLX may not be the predominant biotransformation product in these fish species. Regardless of by-product, Lister et al. (2009) found that FLX was rapidly metabolized in rainbow trout hepatocytes, which they suggest resulted in the observed increasing toxicity with increased exposure time. Ultimately, this highlights the importance of determining relevant metabolites as well as utilizing the appropriate fish species for toxicity studies.

Effects were also noted on growth during the larval stage and found that developmental time to swim-up was significantly affected in lake trout and northern pike, mortality was

significantly increased in the 125 and 500 μg/L treatment groups for both trout species, and biphasic-type responses were noted for fork length in both trout species, although in opposite directions. Comparably, FLX exposure led to a significant drop in weight/decreased growth in adult goldfish and fathead minnows and could therefore also impair growth in developing ELS fish as well (Corcoran et al., 2010; Mennigen et al., 2010). It stands to reason that any pathways that may impact adult fishes are also present in the developing embryo and larvae and are capable of being disrupted by FLX exposure. However, while affected pathways may be present in both the ELS and adult fish, the functional outcome of each pathway may differ between life stages as the nervous system continues to develop (Herculano and Maximino 2014). Moreover, the ability of FLX to interfere with metabolic and developmental processes could explain the outcomes on both growth and developmental time, in particular when developing fish are exposed during embryogenesis, as seen here. Therefore, if larvae are unable to appropriately utilize their energy stores (*i.e.* the yolk), they will be delayed in their transitioning to the next life stage and this, as previously stated, could affect their fitness once winter occurs and growth and development slows in northern latitudes. And, while high mortality did occur in lake trout and rainbow trout, these responses only occurred at 125 and 500 μg/L, which are two to three levels of magnitude higher than reported environmental concentrations.

In larval fish, many organs are still developing as yolk sac absorption, transition to exogenous feeding, and metamorphosis into fry/juveniles occurs, which is denoted as the time when organogenesis is complete (Sehonova et al., 2017). Lake trout and rainbow trout are both fall spawners, and therefore, a shift in degree-day developmental time or size could mean a decreased time span for physiological advancements before winter commences and could also result in a longer delay due to the cooler water temperatures. This could then put them at a

disadvantage over winter and decrease their fitness, especially for lake trout, where some effects could manifest as an almost two-week impact on hatching time (Table C2. S1). Conversely, pike are theorized to undergo fast development as an evolutionary predatory strategy compared to their commonly spring spawning prey species, and where earlier hatching and the associated decreased fork length could reduce their chances of adequate growth and development in summer months as well (Jönsson et al., 2011). However, pike appeared to be relatively tolerant to the apical endpoints analyzed here and it is not likely that environmental concentrations of FLX would affect these endpoints.

3.6.3 ***Histopathology***

Once FLX is taken up by the gills and distributed in the fish body, it does not exhibit the first-pass effect resulting from oral exposure, which results in a larger fraction of the contaminant reaching internal targets and inducing effects (Kreke and Dietrich 2008). Fish are likely responding to the parent compound that is taken up across the gills before it makes its way to the liver, where it is metabolized by the cytochrome P450 superfamily, an enzyme system that is highly conserved in vertebrate species, which is hypothesized to result in the creation of norFLX as a by-product. In turn, the creation of norFLX could mean that exposure to FLX could compromise further detoxification by inhibiting cytochrome P-450 isoenzymes (Kreke and Dietrich 2008). This could also result in potential effects on steroid metabolism and hormonal homeostasis (Kreke and Dietrich 2008). The liver is a major target organ for many environmental contaminants and is the major organ for energy storage and intermediary metabolism. While various studies have analyzed the effects of FLX on mammalian liver, very few have assessed, histologically, the outcomes on fish liver after long-term exposure to FLX.

However, mammalian studies suggest that FLX has the potential to cause hepatotoxicity and lead to transcriptional changes in the liver that activate the NLRP3 inflammasome and increase the production of ROS via transcriptional factor NF- κB (Zlatkovic et al., 2014; De Long et al., 2017).

Analysis of HSI found a decrease in liver size in lake trout, which is contradictory to reports by Mennigen et al. (2010) that found an increase in HSI in goldfish after exposure to 54 and 540 ng/L FLX where food intake and weight gain were significantly inhibited in the 54 ng/L treatment group and metabolic changes, especially with regards to glucose metabolism, were noted in the 540 ng/L treatment group. Conversely, while lake trout exhibited a decreased HSI, all species trended towards higher HVI, which suggests smaller hepatocyte size, and which can be noted in the histological examination of northern pike livers that displayed an obvious decrease in size in terms of lipid storage. This is in accordance with a companion study in this project which assessed proteomic and metabolomic outcomes of FLX in rainbow trout and white sturgeon (*Acipenser transmontanus*) as well as others (Alcaraz et al., personal communication; Craig et al., 2014). Results found that FLX exposure causes a fasting-type response that lead to metabolic disruption (Alcaraz et al., personal communication; Craig et al., 2014). Alcaraz et al. (personal communication) found that pathways associated with bone, skeletal, and notochord development, as well as morphogenesis and development, multiple metabolic processes (including those associated with lipid and carbohydrate metabolism), and cell growth were affected in rainbow trout exposed to 125 μg/L FLX. Disruption in genes associated with lipid and cholesterol metabolism/transport, gluconeogenesis and lipogenesis, as well as pathways associated with stress and inflammation response have also been found in zebrafish (Craig et al., 2014). One study that investigated the hepatic microRNA profile of zebrafish exposed to FLX

found that exposure seemed to mimic a fasting response that downregulated pathways associated with adipogenesis, cholesterol biosynthesis, and triacylglycerol synthesis, which were similar to some of the pathways established in goldfish (Mennigen et al., 2010; Craig et al., 2014). Craig et al. (2014) found that goldfish displayed a significant impact on glucose metabolism at 0.5 μg/L, which could be due to the presence of serotonergic transporters in the liver. They also offered a hypothesis; in mammalian models, both a serotonin transporter and receptor are found on the surface of hepatocytes, which are mediated by a platelet-derived signaling cascade involved in liver regeneration. Furthermore, the presence of the serotonin transporter may become blocked by FLX and lead to liver disfunction (Craig et al., 2014). They also hypothesized that a down-regulation of anabolic pathways after FLX exposure suggests that energy from food that is ingested is not being efficiently assimilated into the necessary metabolites for usage and this poor energy assimilation can have direct consequences on egg production, fecundity, and overall fitness (Craig et al., 2014). Combined, adverse outcomes on one or all of the aforementioned pathways have the ability to lead to cascade effects and result in overall reduced fitness in the species studies in here, which is particularly relevant for rainbow trout, a species that traverses long distances to reach spawning grounds (Washington Fish and Wildlife Department, 2008). Hence, impacts on metabolism or energy storage could have detrimental impacts.

The decreased amount of lipid in the liver of northern pike is a worrisome observation that warrants further investigation, especially due to the otherwise relatively and seemingly resistant status of northern pike in the other endpoints that were assessed here, and which one-time environmental samplings would miss (*i.e.* developmental time). However, it is important to remember that HVI was affected in the 125 μg/L treatment group, which is considerably higher than reported environmental concentrations. Regardless, further research is required to determine

if lower levels of FLX could result in a similar decrease in fitness, could lead to decreased ability and desire to catch prey and an increased susceptibility to infections considering the negative events that starvation can lead to with regards to fish immunity (Agius and Roberts 1981; Kreke and Dietrich 2008; Martin et al., 2010). Therefore, a negative effect on the body condition of fish, especially one which affects pathways already rooted in the immune axis like those that interfere with the serotonin pathway, have the ability to have negative implications for individual and population level fish health.

3.7 Conclusions

Anti-depressants are one of the top 7 leading classes of prescription medications used in Canada for people aged 6 to 79, and SSRIs are a predominant class of neuromodulatory drugs used in anti-depressant treatment (Rotermann et al., 2014). The high prescription rates and the requirement of chronic dosing schedules of SSRIs such as FLX result in the continuous release of these chemicals through municipal effluents into surface waters, which may be of significant concern for native fish species. The results found herein suggest that FLX has the potential to negatively impact growth and development of important fish species and, in some cases, at environmentally relevant concentrations. It also highlights the need for further, more detailed research on specific biochemical aspects of FLX exposure in fish.

We found that there were apparent species-specific differences so that no species responded similarly enough to be able to apply blanket conjectures. Moreover, there seemed to be no consistent trend in sensitivity amongst life stages although, in rainbow trout for example, it did seem that the approximate time of gill development was correlated with significant

mortalities. Regardless, evidence of exposure and effect were noted for all species and life stages, and rainbow and lake trout exhibited some of these events at environmentally relevant concentrations. Due to the implications that this brings forward, it will be particularly important for future research to further delve into the specific mechanisms that FLX imparts on non-mammalian species, especially to further confidence on the predominant metabolite.

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Chapter 4 **: General discussion**

4.1 Introduction

Recent years have shown an increased public concern for the integrity of freshwater and marine environments. This is especially true in Canada which hosts thousands of kilometers of rivers, more than 2 million lakes, and three oceanic coasts (Brownscombe et al., 2014). A major area of concern is the degradation of such environments due to pollution and the impacts that such deterioration may have on fishes, which hold great economic, recreational, and cultural significance to many Canadians. Unfortunately, increasing production of chemicals and their eventual introduction into the environment is threatening the health of aquatic ecosystems in Canada. While many chemicals have been adequately characterized in the environment and biota, there remains a large group of contaminants, commonly referred to as emerging contaminants (EC), which are lacking knowledge regarding their effects on the ecosystems they will eventually enter as well as the non-target organisms they may induce effects in. Chemicals including silver nanoparticles (Ag NPs) and fluoxetine (FLX), which were used in this thesis, are commonly identified ECs that have been quantified in surface waters in North America and ones which developing evidence suggests may lead to adverse and long-lasting effects in wildlife. For many ECs, there is not a good understanding of the extent of their hazards and the potential risks they may cause in fish, especially when exposure coincides with sensitive early life stages (ELS). And, while there are some data that exist on the effects of ECs in fish, it is mostly limited to tropical and sub-tropical laboratory model species, which are widely preferred due to their ease of culturing, succinct life stages, and availability (Powers et al., 2011). Unfortunately, such species may not be particularly relevant to North American ecosystems. Fishes native to the predominantly temperate or cold Canadian freshwater systems commonly exhibit longer life spans, are slower to develop, and grow much larger than more commonly utilized standard

laboratory species. With >28,000 fish species worldwide, there are undoubtedly important genetic, physiological, and ecological discrepancies between commonly used species and species native to colder climates.

Many standard toxicity tests are completed over short time spans and use unrealistically high exposure concentrations, which may not adequately represent exposures that typically occur in the environment. Longer-term exposures often encompass multiple life stages at low concentrations, which are conditions that are more representative to environmental circumstances. The aim of this thesis was therefore to generate novel data on the long-term impacts of an engineered nanoparticle and a pharmaceutical on the early development of three species with commercial, recreational, and aboriginal (CRA) importance when exposed immediately after fertilization and spanning embryonic, larval, and fry life stages. Specifically, the objectives of the present study were to 1) determine the potential effects that two emerging contaminants of concern in Canada, silver nanoparticles and fluoxetine, impart on the ELS of three Canadian fish species for which little data are available; 2) elucidate species and life stage differences in sensitivity upon exposure to these chemicals; and 3) determine multi-life stage impacts that may be missed when investigating individual life stages only.

4.2 Canadian Fish Species as Relevant Species for Toxicity Tests

Recently, changes to the Canadian *Fisheries Act* included a clause that "no person shall carry on any work, undertaking or activity that results in serious harm to fish that are part of a commercial, recreational, or Aboriginal fishery, or to fish that support such a fishery" (Fisheries Act. R.S.C. 1985. c. F-14). This clause denotes the importance and interconnectedness that many

fish species have in supporting the environmental integrity of ecosystems. Therefore, the species selected for the present study were chosen due to their CRA status. The first species, rainbow trout (*Oncorhynchus mykiss*), was chosen due its extensive use in aquatic research as well as it's classification as a keystone species and as a fishery species of CRA interest (Kenchington et al., 2013). A keystone species is thus defined by the Fisheries and Oceans Canada (FOC, previously the Department of Fisheries and Oceans) as a species that has an important role on ecosystem structure and functioning that is disproportionate to their biomass or abundance in the ecosystem (Kenchington et al., 2013). Further, these species exhibit a top-down influence on lower trophic levels and prevent species at lower levels from monopolizing critical resources and thus disrupting the delicate balance of diversity (Kenchington et al., 2013). The second species, lake trout (*Salvelinus namaycush*), was chosen as both a CRA species and apex predator (Kenchington et al., 2013). Apex predators act as indirect support functions to other CRA species by keeping mesopredators such as perch (*Perca flavescens*), northern crayfish (*Orconectes virilis*), and various molluscs in check and thus controlling the food web by maintaining the balance of key prey species (Kenchington et al., 2013). Kenchington et al. (2013) also suggest that apex predators may have unanticipated impacts on trophic cascades involving important processes such as carbon sequestration, disease dynamics, control of invasive species, and biogeochemical cycles. The third species, northern pike (*Esox lucius*), was also chosen as both an apex predator with similar aspects as those mentioned for lake trout, but with a significantly different biology/ecology (Kenchington et al., 2013).

4.3 Effects of ECs on the Early Development of Three Fishes

This thesis aimed to investigate the impacts of two ECs on the early survival and development of three CRA species. Throughout this endeavour, I was able to uncover evidence which suggests that the ELS of North American fishes differ in their sensitivities to different ECs. Furthermore, while many studies suggested that ELS fishes are the most susceptible to toxic insults, they rarely attempted to distinguish which of the three main ELS (*i.e.*, embryo, larva, fry) are the most sensitive. Based on findings from Chapter 2 of this thesis, it appears that the sensitivity of rainbow trout, lake trout, and northern pike largely depends on the developmental stage of the fish as well as the toxicokinetics and toxicodynamics of the chemical. By using individual life stage data for Ag NP exposures, I found that embryos appeared to be the most sensitive life stage in rainbow trout due to the greater number of endpoints affected as well as the lower threshold concentrations, or lowest observable adverse effects concentrations (LOAECs), needed to induce biological effects (Table 4.1). However, in lake trout and northern pike, embryo and larval life stages appeared to be equally impacted, although a greater number of endpoints was affected in northern pike at the embryo stage as well, even though the concentration did not change. For FLX, I was able to support this conjecture by determining that the larval life stage was the most sensitive. Accordingly, the larval stage is the approximate time of early gill development and therefore a time of increased capacity for drug metabolism. This stage had more pronounced effects than the embryonic or fry stage following FLX exposure, since there was a larger number of endpoints and lower effect concentrations in the trout species, although northern pike did not display this trend (Table 4.2). Although northern pike were impacted at a relatively low level for Ag NPs, the characteristics of the response curves need to be analyzed further. Or, perhaps studies which include a wider variation of concentrations need

to be completed, as it was sometimes difficult to determine if the effects were biologically relevant or due to random error. Many endpoints did not display a consistent exposure-response curve and the variation was sometimes difficult to interpret as the lowest concentration was often significant whereas other concentrations were not. However, northern pike also demonstrated a consistently increased developmental time in all treatment groups when exposed to FLX and, while only 2 treatments displayed statistical significance, the shift in degree days that was noted could lead to a delayed hatching of upwards of one and a half weeks, which would undoubtedly impact fitness. Regardless, across most endpoints and similar to past studies (e.g. Elonen et al., 2009), both Chapters 2 and 3 suggest that NP seem to be fairly resilient to the effects of long-term exposure to ECs during the ELS. However, it is important to note that, due to culturing issues, certain endpoints were unable to be measured, and which may have shown particular sensitivity. Furthermore, it appeared that rainbow trout were a fairly consistent representative species to the other species used here in terms of sensitivity to both Ag NP and FLX for all three life stages. This is welcome news considering the prevalence of rainbow trout in aquatic toxicology studies and their use to extrapolate to other CRA species for the purpose of regulations and guidelines.

Table 4.1: Comparison of the number of endpoints and LOAECs amongst the three species and three life stages analyzed for Ag NPs. The data here suggest that the embryonic stage was the most sensitive for all three species with the most endpoints being impacted and at lower concentrations.

	Rainbow Trout		Lake Trout		Northern Pike	
	Endpoints	Concentration (nM)	Endpoints	Concentration (nM)	Endpoints	Concentration (nM)
Embryo	3	0.10	2	3.00	3	0.10
Larval	2	10.0	2	3.00	2	0.10
Fry	2	30.0	1	10.0	0	-

Table 4.2: Comparison of the number of endpoints and LOAECs amongst the three species and three life stages analyzed for FLX. The data suggests that the larval stage was the most sensitive stage for both trout species. However, northern pike appeared relatively insensitive to the endpoints analyzed here.

	Rainbow Trout		Lake Trout		Northern Pike	
	Endpoints	Concentration (µg/L)	Endpoints	Concentration (µg/L)	Endpoints	Concentration (µg/L)
Embryo	1	2.00	0	-	1	125
Larval	2	0.50	3	0.50	1	125
Fry	1	125	1	2.00	1	125

The results of this thesis research suggest that certain North American fishes may be susceptible to ECs of concern at concentrations that are environmentally relevant, and which may be eliciting effects that could have long-term, population- and ecosystem-level implications. By analyzing long-term, multi-life stage data, I was able to determine that survival across life stages was sometimes impacted even while individual life stage survival may not have been. Realistically, most ELS fishes are relatively confined to the breeding locations where they hatch until they have grown considerably larger. Moreover, the majority of chemical exposures are not one-time. Hence, current guidelines based on single life stage and short-term exposures may not be able to fully encompass the sensitive nature of ELS fishes and may not be sufficiently protective of a species that is being consistently exposed. Nevertheless, some limitations of the present study were the difficulty in obtaining a larger sample size for gametes. Unfortunately, there were limitations with the survival analysis in the present study that make more accurate predictions difficult and further studies should aim to more adequately characterize the survival dynamics of these species, as well as other laboratory species. To the best of the author's knowledge, this was the first study to analyze multi-life stage survival dynamics in North American fishes to two ECs of concern.

4.4 Implications for Canadian Ecosystems

Fish are one of Canada's most profitable renewable resources and expenditures related to recreational fishing alone totaled approximately 2.5 billion dollars in 2010 (Tufts et al., 2015). The appeal of commercial, recreational, and cultural fishing is particularly vital for rural and northern communities, which profit more substantially than others due to the stimulation of local

economies (Tufts et al., 2015). This concern is further exacerbated when taken in combination with past studies that have noted that rainbow trout, lake trout, and northern pike populations had been decreasing in the Pacific, Arctic, Hudson Bay, and Atlantic drainage basins (Post et al., 2002). The areas that were assessed in this study were noted to be adjacent to urban areas and the Canada-USA border and were thought to be due to habitat deterioration, along with overfishing (Post et al., 2002). Regrettably, similar reviews on the current status of recreational fisheries and their species have not been published recently, which makes conjectures about the stability of these populations difficult (Post 2012). However, not only are fishes important to the economy, they are also crucial to the livelihood of many Northern communities. For example, it was estimated that approximately 86% of the residents of the Dehcho First Nation region, Northwest Territories, Canada reported eating fish at least once a day (Guyot et al., 2006). This is particularly relevant due to the increasing reliance on subsistence fishing that can be attributed due to the declining caribou populations across northern Canada (Schuster et al., 2011). Furthermore, many northern and rural communities lack the means necessary to adequately treat wastewater, which is a large source of introduction of many contaminants into the environment, and particularly local ecosystems that are utilized for these sustenance fisheries.

The majority of the Canadian population is located in the lower latitudes of the country along or within close proximity to lakes or rivers (Statistics Canada, 2018). Consequently, as defined by Chu et al. (2003), these are also areas of high stress, which are described as those with high cumulative population, agriculture, and industrial stresses. Interestingly, these regions also commonly coincide with areas of high species conservation priority, high environmental index (which is defined as relatively humid, warm and diverse areas within Canada), as well as biodiversity and species rarity indices, as discussed in Chu et al. (2003). Therefore, the regions

that are likely most susceptible to the negative impacts of ECs are the regions that are most likely exposed to the highest concentrations of ECs.

4.5 Recommendations for Future Research

This research, to the best of the author's knowledge, is the first of its kind to assess long-term, multi-life stage exposures to ECs on North American fishes. However, it was mainly intended as a screen and, while it was successful in laying groundwork for multi-life stage and non-standard species toxicity testing, future studies should take into consideration some gaps in the present work and, hopefully, forthcoming research will be able to contribute to a more robust understanding of the impacts of ECs on native Canadian fishes in long-term, ELS studies.

Within the scope of the larger project in which this study was a part, two parallel studies were completed in order to assess the biochemical mechanisms of action of the present chemicals in the same fish species. Alcaraz et al., (personal communication) and Eisner et al., (personal communication) characterized molecular toxicity pathways as well as transcript abundances of selected target genes, respectively, in liver and gonads across multiple CRA species. Preliminary results from these studies suggest that multiple pathways that may have implications for various apical outcomes are present. It will be interesting to bring the findings from all of these studies together and attempt to characterize the toxicity pathways of Ag NPs and FLX in fish in order to gain a more complete view of the connection between *in vivo* and *in vitro* toxicity tests. Moreover, this combined information will hopefully aid in generating a model that is able to more accurately and reliably extrapolate between *in vivo* and *in vitro* results.

Due to the scale of the present study and larger project as a whole in terms of number of species and chemicals, as well as the required length, there are several analyses that could be undertaken in order to bolster the findings in my thesis. The larger project aimed to assess impacts of five chemicals (including hexabromocyclododecane, short chain chlorinated paraffins, and ethynylestradiol, along with Saskatoon wastewater effluent) that are commonly found in wastewater effluents during long-term exposures. The quantification of Saskatchewan surface waters or, at least, Saskatoon wastewater effluent, for the ECs studied would provide excellent insight into the determination if these ECs are quantifiable in Saskatchewan waters. Finally, a more in-depth analysis of the survival curves generated here in efforts to better characterize (and potentially predict) survival dynamics in the case of these species to the studied ECs and could, when combined with current literature in the field, provide an excellent model for policy makers and legislators. Unfortunately, due to time constraints as well as differences in metabolic capacities, and therefore, across life stage responses, this analysis was omitted.

As mentioned, samples are available for other chemicals in a few of the species used here. Aside from data presented in this thesis, wastewater effluent and ethynylestradiol exposures were conducted in rainbow trout, wastewater effluent in lake trout, and all chemicals in northern pike. In all exposures, where possible, multiple samples from each life stage were preserved by snap freezing or submerging in formalin for future further analysis. Specifically, body burden analysis would be useful to provide exposure and uptake data for the study chemicals. This could then help to lend insight into the biochemical tendencies, such as the distribution and metabolism, especially FLX where it is also particularly important to determine the predominant metabolite that elicits effects as well as to further determine the implications that serotonin plays in fish

systems. If we are able to understand the toxicokinetics of this EC in fish, we may be better able to understand the effects it causes, including effects on behavioural endpoints.

Further histological work could also undertake complete decalcification of the juvenile fry that were unexpectedly quite boney, and which resulted in difficulties sectioning. Alternately, juveniles could also be dissected for relevant tissues to further analyze impacts on specific tissues, which would greatly expedite the histopathological process, as well as determine impacts on sexual differentiation, where possible. However, others should aim to carry on the exposure of fishes for a slightly longer time span in order to ensure ability to dissect out whole organs in order to assess apical outcomes associated with sexual differentiation, liver and gill histopathology, and any other endpoints on relevant tissues. In the current study, many of the fishes and their organs were too small to be dissected out, especially in northern pike, which greatly complicated histological analysis. It would also be interesting for future research to experiment with different staining methods for the histological analysis in order to include endpoints associated with glycogen storage and mucous secretion (by using periodic acid-Schiff reaction and Alcian Blue stains), fibrosis (using Masson's Trichome), as well as immunohistochemistry for relevant tissues.

The present study aimed to assess the differences between life stages but, due to the experimental design, it was not possible to run individual life stage assessments separately from the whole. The life stage exposures in the present studies were conducted continuously and therefore fry had been exposed since fertilization and not just since transitioning. It would therefore be informative to conduct further studies that analyze the impacts of FLX and Ag NPs on the individual stages and determine if the effects are similar if the life stages were handled independently or if the impacts are due to the cumulative exposure. Furthermore, the exposures

herein dealt with a large number of organisms as well as chemicals and concentrations, which made it impossible to fertilize the fish eggs in exposure solutions. An interesting piece to the present EC puzzle would be to study the impacts of these chemicals on initial fertilization in terms of effects on sperm mobility, energetic, and fertilization potential, as well as on egg permeability.

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APPENDICES

Supplementary materials submitted with manuscripts are included herein. Figures and table numbers are presented in Cx. Sy format such that ‘Cx’ indicates chapter number and ‘Sy’ indicates figure or table number.

Appendix A: Water Quality

Facility Water

Key parameters measured for exposure and ATRF water ranges (in brackets) included dissolved oxygen (>90%), pH (7.6 – 7.9), conductivity (220 µS/cm), alkalinity (~130 µg $CaCO_3$/L), ammonia (< 0.02 µg/L), nitrates (< 1.5 µg/L). Water quality was measured using a YSI Quatro Professional Plus multiparameter field cable (Yellow Springs, USA) or testing kits (LaMotte, Chestertown, USA).

Solvent Controls

Ethanol was used a solvent control for both trout exposures for FLX. However, due to rising concerns of its suitability as a solvent, mostly owing to the presence of a biofilm that appeared and had slight effects on water quality, acetone was used as the solvent for northern pike. Acetone controls were used as a comparison, where possible, for northern pike exposures until culturing issues lead to the failure of the acetone control after swim-up, after which the DMSO control (which was the control for hexabromocyclododecane and short chain chlorinated paraffin exposures) was utilized for comparisons instead.

Appendix B: Supplementary Apical Data

Table C2:S1: Developmental endpoints of three fish species from fertilization to hatch (embryonic life stage) after exposure to increasing concentrations of Ag NP. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Malformations for *E. lucius* were omitted due to suspected confounding factors (see discussion).

Species	Ag NP Concentration (nM)	Fertilization Success (%)	Degree Days to Hatch	Percent Morality	Percent Malformations	Length
Salvelinus namaycush	0.00	93.7 (90.3, 100)	573 ± 3.57	6.61 ± 1.29	5.44 ± 0.458	20.8 (20.0, 21.0)
	0.10	93.7 (89.7, 100)	607 ± 10.01	4.23 ± 2.50	13.0 ± 3.79	21.0 (20.4, 21.5)
	0.30	94.7 (94.6, 100)	600 ± 13.4	3.44 ± 1.83	2.71 ± 1.80	21.0 (20.5, 21.1)
	1.00	98.4 (93.6, 100)	621 ± 0.00	8.96 ± 1.25	4.96 ± 1.15	20.3 (19.8, 21.0)
	3.00	98.2 (96, 100)	671 ± 9.75*	8.40 ± 3.26	3.07 ± 1.53	20.3 (19.5, 21.0)
	10.0	98 (94.4, 100)	645 ± 6.13*	5.55 ± 0.655	2.10 ± 0.0148	19.5 ***** (19.0, 20.0)
	30.0	100 (98.6, 100)	654 ± 24.7*	13.3 ± 1.71	6.40 ± 0.947	20.0 (19.5, 21.0)
Oncorhynchus mykiss	0.00	89.0 (75.0, 98.5)	399 (390, 414\0	28.0 ± 12.2	3.50 ± 1.50	20.5 (18.5, 22.0)
	0.10	97.9 (93.1, 98.2)	413 (391, 429)	37.5 ± 6.46	.958 ± 0.590	15.0 * (14.0, 17.0)
	0.30	96.5 (96.0, 97.4)	406 (406, 417)	41.1 ± 5.42	1.97 ± 0.30	16.5 * (16.0, 17.0)

	1.00	95.7 (93.6, 97.3)	405 (370, 433)	39.2 ± 2.93	4.75 ±1.80	19.0 (17.8, 20.5)
	3.00	94.8 (94.6, 98.7)	417 (393, 445)	40.7 ± 3.83	2.91 ± 0.620	17.0 * (16.0, 17.5)
	10.0	94.5 (93.2, 99.2)	427 (415, 454)	40.0 ± 14.8	7.69 ± 4.13	18.0 (17.6, 20.0)
	30.0	98.6 (95.1, 98.9)	462* (434, 462)	55.4 ± 20.3	19.0 ± 7.12 *	17.0 (17.0, 18.3)
Esox lucius	0.00	100 (100, 100)	212 (201, 224)	22.5 (14.7, 24.5)	-	11.7 ± 0.116
	0.10	100 (100, 100)	143 * (129, 143)	22.5 (22.5, 28.6)	-	12.1 ± 0.251
	0.30	100 99.0, 100	169 (155, 169)	33.6 (21.9, 37.8)	-	11.1 ± 0.211
	1.00	100 98.7, 100	156 (156, 156)	33.9 (29.4, 39.0)	-	10.6 ± 0.290 *
	3.00	98.8 98.0, 100	147 (147, 159)	37.4 * (34.2, 38.6)	-	10.6 ± 0.156*
	10.0	100 98.8, 100	186 (162, 186)	32.5 (27.5, 32.6)	-	11.1 ± 0.254
	30.0	100 97.6, 100	150 * (136, 150)	31.9 (28.3, 34.2)	-	11.0 ± 0.211

Table C2.S2: Developmental endpoints of three fish species from hatch to swim-up (larval life stage) after exposure to increasing concentrations of Ag NP. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Length measurements for *E. lucius* were omitted due to confounding factors (see discussion).

Species	Ag NP Concentration (nM)	Degree Days to Swim-up	Percent Morality	Length (mm)
Salvelinus namaycush	0.00	381 ± 5.15	34.9 ± 8.94	29.6 ± 0.491
	0.10	431 ± 13.3	21.3 ± 3.10	29.2 ± 0.335
	0.30	333 ± 10.8	37.2 ± 14.9	29.7 ± 0.413
	1.00	390 ± 3.56	7.52 ± 4.33	28.9 ± 0.363
	3.00	488 ± 17.7 *	5.04 ± 2.89	28.2 ± 0.331*
	10.0	435 ± 34.19	2.57 ± 1.52	28.6 ± 0.250
	30.0	467 ± 24.3	25.5 ± 8.85	29.8 ± 0.266
Oncorhynchus mykiss	0.00	202 ± 4.70	23.2 ± 13.7	29.7 ± 0.339
	0.10	198 ± 10.8	5.05 ± 0.448	30.0 ± 0.259
	0.30	217 ± 3.60	21.3 ± 17.1	29.3 ± 0.306
	1.00	237 ± 22.0	58.5 ± 23.0	28.5 ± 0.493
	3.00	207 ± 8.15	38.2 ± 21.2	30.5 ± 0.361
	10.0	198 ± 11.6	13.2 ± 7.04	31.1 ± 0.340*
	30.0	252 ± 24.0*	40.0 ± 18.1	27.7 ± 0.526 *
Esox lucius	0.00	120 (118,124)	7.65 ± 1.16	-
	0.10	203 * (191, 213)	19.6 ± 4.99	-
	0.30	167	6.26 ± 0.354	-

		(141, 169)		
	1.00	167 (167, 195)	18.1 ± 0.082	-
	3.00	176 (162, 176)	12.6 ± 4.61	-
	10.0	141 (141, 155)	9.86 ± 0.764	-
	30.0	179 (155, 180)	21.4 ± 2.02 *	-

Table C2.S3: Developmental endpoints of three fish species from swim-up to approximate sexual differentiation (fry life stage) after exposure to increasing concentrations of Ag NP. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Mortality for *E. lucius* were omitted due to confounding factors and HSI omitted due to small fish size.

Species	Ag NP Concentration (nM)	Percent Morality	Length (mm)	Hepatosomatic Index
Salvelinus Namaycush	0.00	47.4 ± 11.0	62.0 ± 1.47	1.20 (1.18, 1.29)
	0.10	11.5 ± 4.30	-	
	0.30	12.3 ± 6.71	-	
	1.00	37.2 ± 31.4	54.9 ± 0.435 *	1.21 (1.04, 1.38)
	3.00	11.7 ± 3.73	56.3 ± 0.498 *	1.21 (1.02, 1.46)
	10.0	7.82 ± 1.43	-	
	30.0	4.99 ±1.86	-	
Oncorhynchus mykiss	0.00	24.5 ± 10.2	52.2 ± 0.496	1.15 ± 0.0592
	0.10	33.0 ± 13.9	-	
	0.30	60.0 ± 16.9	-	
	1.00	64.2 ± 23.5	-	
	3.00	54.4 ± 10.7	-	
	10.0	53.0 ± 25.9	52.4 ± 0.662	1.21 ± 0.0753
	30.0	51.7 ± 24.6	53.7 ± 0.785	1.59 ± 0.0851*
Esox Lucius	0.00	-	20.1 ± 0.554	-
	0.10	-	-	-
	0.30	-	-	-
	1.00	-	-	-
	3.00	-	-	-
	10.0	-	21.5 ± 0.483	-
	30.0	-	-	-

Table C3:S1: Developmental endpoints of three fish species from fertilization to hatch (embryonic life stage) after exposure to increasing concentrations of FLX. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Malformations for *E. lucius* were omitted due to suspected confounding factors (see discussion).

Species	FLX Concentration (μg/L)	Fertilization Success (%)	Degree Days to Hatch	Percent Mortality	Malformations (%)	Length
Salvelinus namaycush	0.00	95.4 ± 1.60	582 (570, 582)	6.61 ± 1.29	8.67 ± 2.10	19.25 (17.9, 20.3)
	0.50	97.2 ± 1.51	585 (534, 585)	5.88 ±1.98	5.01 ± 1.27	20.5 (16.4, 21)
	2.00	93.6 ± 3.26	590 (580, 590)	5.87 ± 4.32	7.04 ± 3.86	20.0 (19.0, 21.0)
	8.00	94.0 ± 4.27	576 (542, 591)	5.68 ± 2.24	12.0 ± 1.01	19.0 (19.0, 20.6)
	32.0	96.4 ± 1.81	562 (542, 587)	5.42 ± 3.48	8.20 ± 3.01	19.8 (18.6, 20.0)
	125	99.4 ± 0.58	580 (566, 58)	10.3 ± 4.35	19.4 ± 4.52	19.5 (18.3, 20.0)
	500	97.1 ± 1.46	565 (565, 589)	3.99 ± 2.41	17.0 ± 3.47	18.5 (18.0, 19.1)
Oncorhynchus mykiss	0.00	97.7 ± 1.63	446 (446, 446)	35.7 ± 11.1	8.68 (3.49, 13.9)	20.5 (18.5, 22.0)
	0.50	97.6 ± 1.04	444 (444, 456)	21.7 ± 0.685	0.862 (0.00, 2.63)	18.5 * (18.1, 19.4)
	2.00	97.5 ± 1.79	423	18.7 ± 3.86	0.671 (0.00, 0.848)	16.5

			(412, 435)			(14.0, 17.0)
	8.00	94.8 ± 3.76	391 (391, 391)	12.7 ± 2.30	1.21 (0.00, 2.56)	19.5 (18.0, 20.0)
	32.0	98.6 ± 1.40	396 (396, 423)	18.1 ± 6.91	0.00 (0.00, 0.00)	19.0 (18.0, 19.0)
	125	98.6 ± 0.330	448 (423, 464)	22.0 ± 10.0	0.00 (0.00, 12.3)	17.0 * (16.0, 18.8)
	500	97.4 ± 1.81	458 (430, 470)	17.7 ± 10.8	0.926 (0.00, 3.18)	16.8 * (15.5, 19.3)
Esox Lucius	0.00	98.9 (97.6, 100)	214 (209, 234)	40.2 ± 9.01	-	11.0 (11.5, 10.8)
	0.50	100 (100, 100)	182 (156, 182)	37.9 ± 6.18	-	12.0 (12.0, 11.0)
	2.00	100 (95.2, 100)	209 (195, 237)	24.0 ± 3.67	-	10.0 (9.5, 11.0)
	8.00	100.00 (100, 100)	173 (145, 173)	40.6 ± 2.35	-	12.0 (11.0, 12.0)
	32.0	97.6 (97.3, 100)	168 (141, 168)	21.8 ± 5.38	-	11.0 (11.0, 12.0)
	125	98.2 (95.1, 100)	155* (140, 157)	23.1 ± 3.26	-	11.8 (11.4, 12.0)
	500	100 (98.7, 100)	132 * (132, 132)	25.1 ± 3.97	-	11.5 (11.0, 12.0)

Table C3.S2: Developmental endpoints of three fish species from hatch to swim-up (larval life stage) after exposure to increasing concentrations of Ag NP. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Length measurements for *E. lucius* were omitted due to confounding factors (see discussion)

Species	FLX Concentration (mg/L)	Degree Days to Swim-up	Percent Mortality	Length (mm)
Salvelinus Namaycush	0.00	411 ± 46.0	34.9 ± 8.94	30.6 ± .0622
	0.50	549 ± 30.3*	52.8 ± 12.9	32.2 ± 0.902
	2.00	424 ± 24.5	49.4 ± 12.5	31.3 ± 0.923
	8.00	383 ± 25.6	75.1 ± 2.71	30.0 ± 1.04
	32.0	294 ± 25.2	61.2 ± 3.90	28.3 ± 0.862
	125	299 ± 19.6	76.7 ± 12.1 *	26.2 ± 0.655 *
	500	-	87.3 ± 1.99 *	-
Oncorhynchus Mykiss	0.00	207 (192, 216)	41.9 ± 13.3	31.0 (29.0, 32.4)
	0.50	307 (215, 319)	41.2 ± 5.70	28.0 (26.5. 30.5)
	2.00	260 (247, 331)	31.8 ± 7.63	28.0 (26.0. 30.0)
	8.00	243 (215, 282)	1.42 ± 0.709	30.0 (29.0. 31.4)
	32.0	269 (206, 272)	8.90 ± 7.27	30.0 (28.5, 31.0)
	125	169 (169, 169)	98.5 ± 0.762 *	-
	500	-	100.0 ± 0.00 *	-
Esox Lucius	0.00	122 (113, 151)	11.9 ± 4.09	-
	0.50	149 (149, 178)	17.1 ± 2.05	-
	2.00	137 (109, 151)	8.77 ± 1.80	-
	8.00	155 (155, 170)	8.92 ± 1.97	-
	32.0	167 (155, 170)	6.69 ± 1.21	-

	125	179 * (179, 194)	10.7 ± 2.41	-
	500	168 (168, 168)	17.7 ± 3.40	-

Table C3.S3: Developmental endpoints of three fish species from swim-up to approximate sexual differentiation (fry life stage) after exposure to increasing concentrations of FLX. For data that passed normality tests, means ± SEM are given. For data that did not pass normality tests, the median is given with 25th percentile and 75th percentiles in parenthesis. Asterisks (*) denote statistical significance ($p \leq 0.05$). Mortality for *E. lucius* were omitted due to confounding factors and HSI omitted due to small fish size.

Species	FLX Concentration (μg/L)	Percent Mortality	Length (mm)	Hepatosomatic Index
Salvelinus Namaycush	0.00	47.4 ± 11.0	62.5 ± 1.26	1.64 ± 0.135
	0.50	29.8 ± 14.8	64.5 ± 1.11	1.54 ± 0.0509
	2.00	18.9 ± 1.11	59.9 ± 1.12	1.2 ± 0.0883*
	8.00	26.7 ± 5.80	-	-
	32.0	54.1 ± 8.28	-	-
	125	21.7 ± 11.7	-	-
	500	-	-	-
Oncorhynchus mykiss	0.00	28.7 ± 11.2	52.0 (50.0, 54.8)	1.20 (0.90, 1.41)
	0.50	32.8 ± 9.95	-	-
	2.00	32.5 ± 14.0	-	-
	8.00	21.7 ± 5.25	52.0 (51.0, 53.0)	1.24 (1.05, 1.40)
	32.0	23.6 ± 11.3	53.0 (50.5, 55.0)	1.16 (0.972, 1.42)
	125	100 ± 0.00 *	-	-
	500	-	-	-
Esox lucius	0.00	-	20.1 ± 0.554	-
	0.50	-	21.2 ± 0.441	-
	2.00	-	-	-
	8.00	-	-	-
	32.0	-	-	-
	125	-	18.6 ± 0.498	-
	500	-	-	-

Appendix C: Histology Protocols

Processing Program

1. 70% Ethanol	1 hour	
2. 70% Ethanol	1 hour	
3. 70% Ethanol	1 hour	
4. 80% Ethanol	1 hour	
5. 95% Ethanol	1 hour	
6. 100% Ethanol	30 mins	
7. 100% Ethanol	30 mins	
8. 100% Ethanol	1 hour	
9. Xylene	30 mins	
10. Xylene	30 mins	
11. Paraplast wax	30 minutes	62°C
12. Paraplast wax	30 minutes	62°C
13. Paraplast wax	10 mins	62°C

Staining Protocol

1. Xylene	2 minutes

2.	Xylene	2 minutes
3.	100% alcohol	3 minutes
4.	100% alcohol	3 minutes
5.	95% alcohol	2 minutes
6.	70% alcohol	2 minutes
7.	Running water (warm)	2 minutes
8.	Distilled water	Two dips
9.	Hematoxylin	5 minutes
10.	Running water (warm)	2 dips
11.	Acid Alcohol	1 dip
12.	Running water (warm)	10 minutes
13.	Distilled water	1 dip
14.	Eosin	3 minutes
15.	Running water	2 dips
16.	70% alcohol	1 dip
17.	95% alcohol	1 dip
18.	100% alcohol	1 dip
19.	100% alcohol	1 minutes
20.	Xylene	2 minutes
21.	Xylene	Up to 1 hour

Appendix D: Representative Malformations

Table C2.S1: Representative photographs of common malformations in rainbow trout embryos after exposure to varying concentrations of Ag NPs.

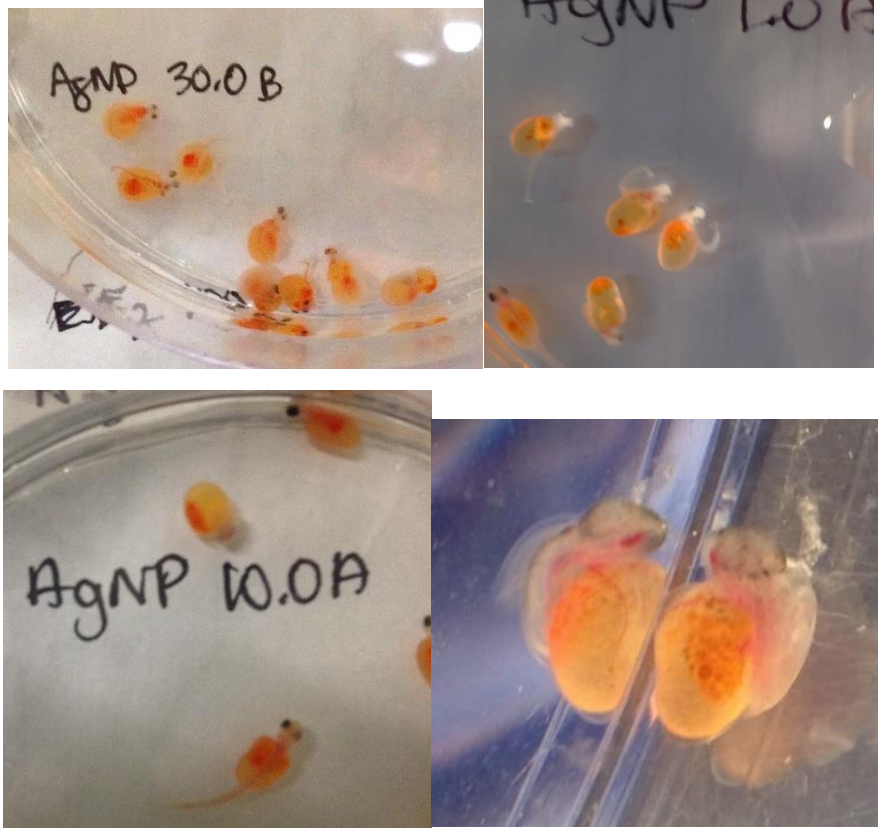
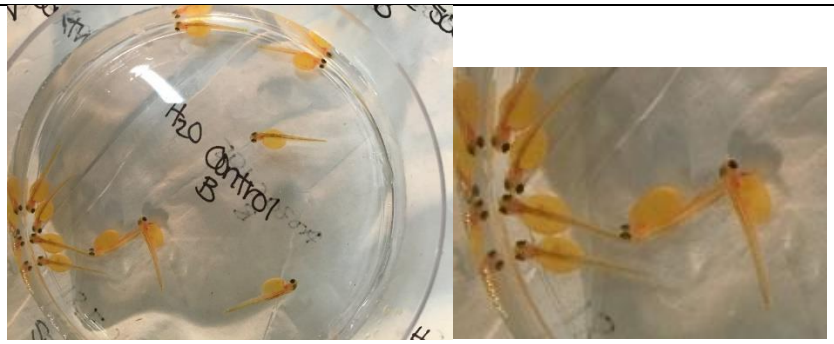
Treatment Type	Sample Picture
Control	
Ag NP	

Table C2.S2: Representative photographs of common malformations in lake trout embryos, larvae, and fry after exposure to varying concentrations of Ag NPs.

Treatment Type	Sample Picture
Control	

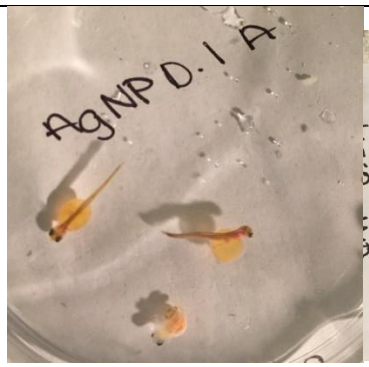
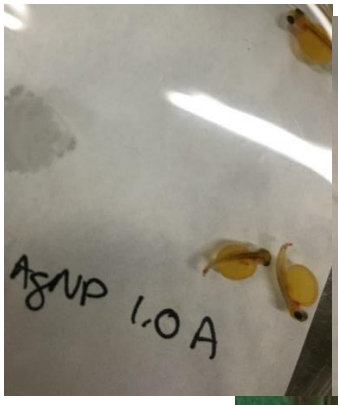
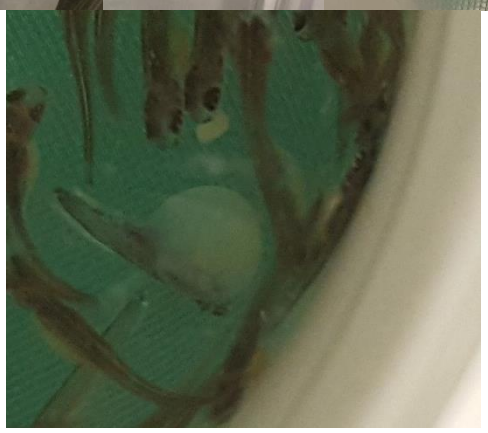
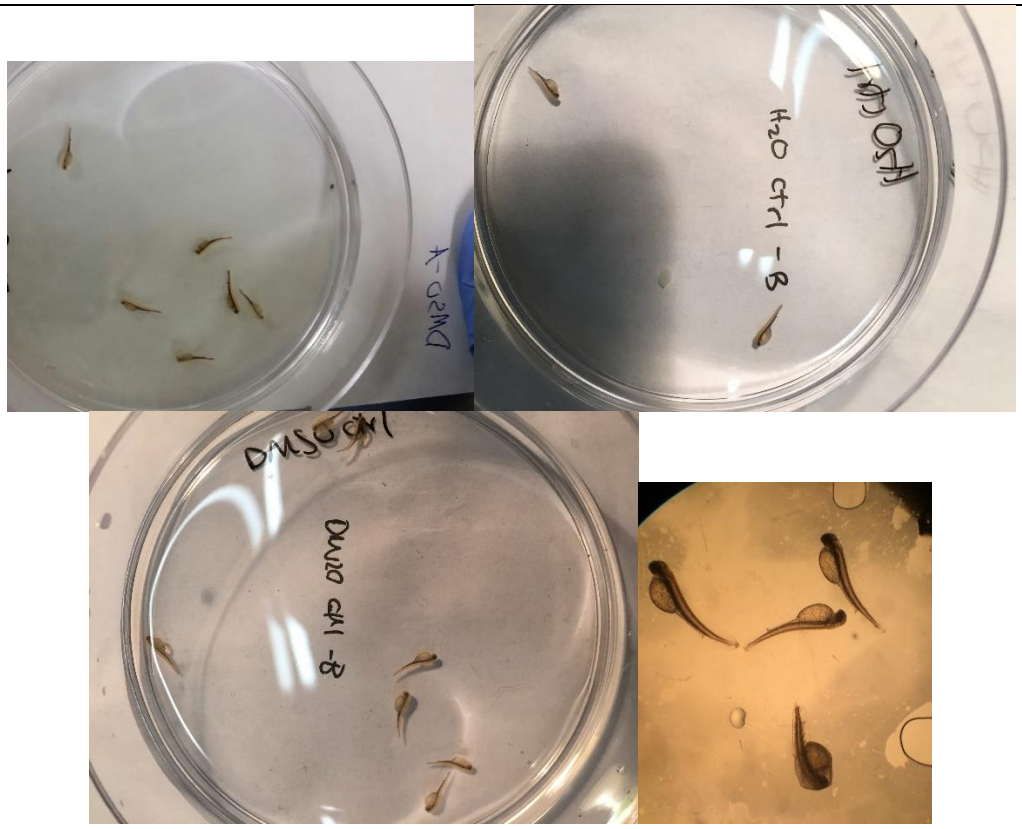
Ag NP	     

Table C2.S3: Representative photographs of common malformations in northern pike embryos after exposure to varying concentrations of Ag NPs.

Treatment Type	Sample Picture
Control	

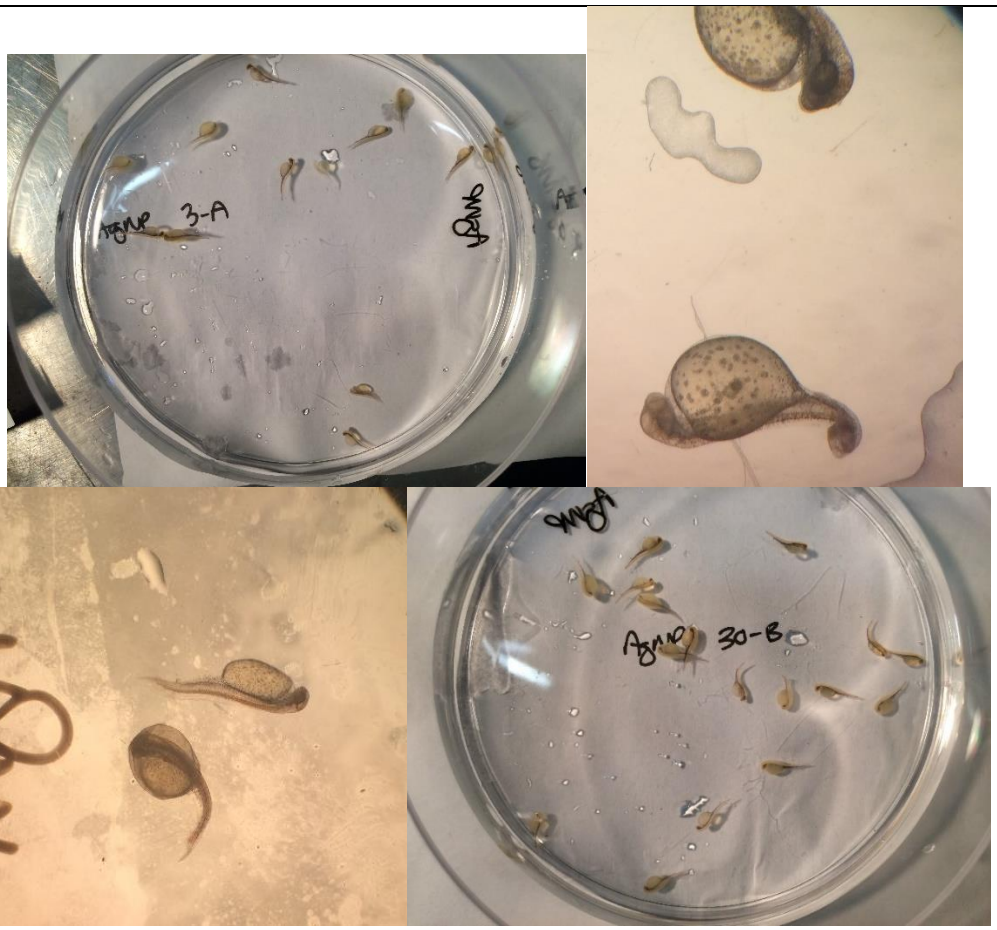
Ag NP	

Table C3.S1: Representative photographs of common malformations in rainbow trout embryos after exposure to varying concentrations of FLX.

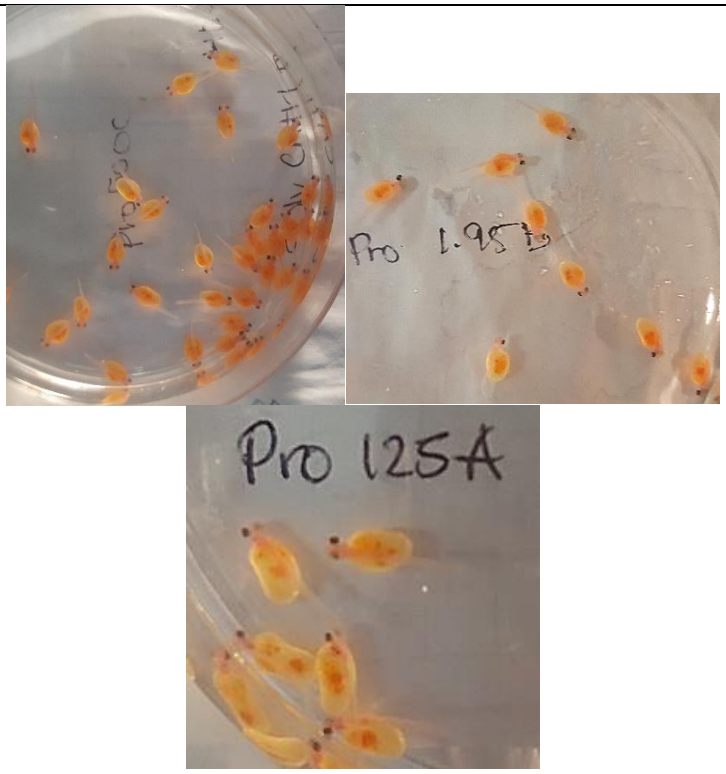
Treatment Type	Sample Picture
Control	
FLX	

Table C3.S2: Representative photographs of common malformations in lake trout embryos after exposure to varying concentrations of FLX.

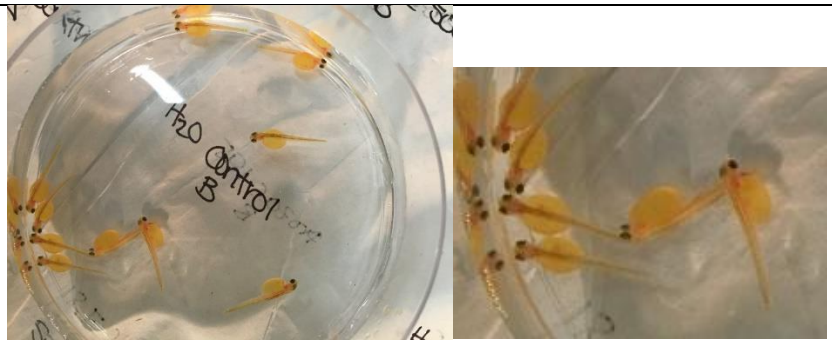
Treatment Type	Sample Picture
Control	
FLX	

Table C3.S3: Representative photographs of common malformations in northern pike embryos, larvae, and fry after exposure to varying concentrations of FLX.

Treatment Type	Sample Picture
Control	
FLX